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CORRECTION

Owing to a mathematical error the values found for the lysine and histidine content of egg albumin were incorrectly stated in the original publication (Calvery, H. O., *J. Biol. Chem.*, **94**, 613 (1931–32)). The values should read: lysine, 3.95 per cent; and histidine, 1.40 per cent.

ON THE FATTY ACIDS ESSENTIAL IN NUTRITION. III*

By GEORGE O. BURR, MILDRED M. BURR, AND ELMER S. MILLER

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After it was demonstrated (1) that a deficiency disease was caused by the lack of fatty acids in the diet, a study of the well known natural fatty acids was undertaken. In the second paper of this series (2) it was shown that none of the saturated fatty acids occurring in hydrogenated coconut oil was effective in curing the disease and promoting renewed growth of the animal. Pure methyl linolate is highly effective in curing sick animals and all oils which contain appreciable amounts of this acid are likewise good.

Since butter (30 per cent oleic acid) gave very poor results at the high level of 300 mg. daily (3 per cent of the diet) it was postulated that oleic acid is entirely negative and the small effects due to butter were due to traces of linoleic acid. But a sample of commercial methyl oleate gave good results and the value of oleic acid was left uncertain. Other common fatty acids which are now being studied are linolenic, arachidonic, and eleostearic. It is the object of this paper to report the results of some studies of these acids.

Diet and Technique

The constant temperature room is now maintained at 26.0° ± 1° the year round. The same cages and diets described in the first paper (1) are used for all work unless specifically stated to be changed. The maintenance diet, Diet 550-B, contains 12 per cent pure casein, 84.1 per cent sucrose, and 3.9 per cent salt mixture

(McCollum Salt Mixture 185) (3). This is supplemented daily
with 0.65 gm. of ether-extracted Northwestern dry yeast, and the
non-saponifiable matter from 70 mg. of highest grade cod liver oil
(Patch) and from 35 mg. of wheat germ oil. All known vitamins
seem to be supplied in excess. The drinking water is distilled and
contains 0.27 mg. of KI per liter.

Rats are weaned when 21 days old and put on the low fat diet.
They must weigh over 36 gm. on weaning day. The weight
curves reach a plateau when the rats are about 150 gm. in
weight and when it has been established that they have reached
their maximum weight and are actually declining slightly they are
used as cures. Positive results are marked by a clearing of the
skin, improvement of hair coat, and renewed growth both in length
and weight. Increase in weight is used as the quantitative meas-
ure of the effectiveness of an oil or fatty acid.

<center>EXPERIMENTAL</center>

Linoleic Acid—In the preceding paper (2) 5 drops daily of methyl
linolate were used. When larger or smaller doses are used (10 or
3 drops) marked differences of rate of response can be seen (Chart
I). By similar studies on oils it has been demonstrated that
maximum effects are reached at the 10 drop level. The pure
methyl linolate was prepared from corn oil by the method of
Rollett (4).

Oleic Acid—The preparation of oleic acid free from appreciable
quantities of contaminating acids presents some difficulties. The
method of Lapworth *et al.* (5) was used for olive oil. This pro-
cedure requires the separation of lead salts, preparation and puri-
fication of barium oleate, and finally esterification and distillation.
This preparation is presumably free from linoleic acid and contains
about 2 per cent palmitic acid, which does not interfere with our
tests.

Since butter gave almost negative results as a curative fat, oleic
acid was also prepared from it. Melted and filtered butter was
saponified and esterified in the usual way and the esters subjected
to fractional distillation. All of the lower fatty acid esters were
removed at 140° and 3 mm. pressure. The residue was saponified
and the oleic acid was purified by the same technique as used for
olive oil. Yield, 75 cc. of methyl oleate from 800 gm. of butter.

The methyl oleate was fed to six rats, three receiving the olive oil acid and three the butter acid. The curves given in Chart II show that no growth resulted from the feeding of these esters over a period of 50 days. The hair coat, skin, and tail showed no improvement. There is no evidence that oleic acid has any curative effect although it may arrest the downward trend of the animals. It seems, therefore, that the slight positive effects noted
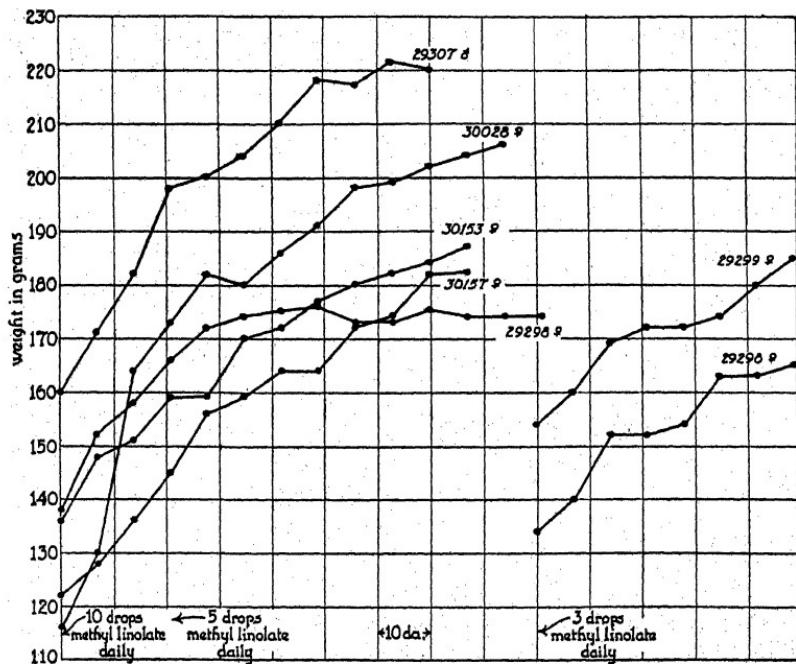
CHART I. Weight curves showing the growth response of rats to different quantities of methyl linolate. Along with the renewed growth there is rapid clearing of the skin.

for butter (2) are due to acids more unsaturated than oleic, probably linoleic.

Linolenic Acid—Although linolenic acid is usually absent from stored fats such as lard and tallow, it may be deposited in the fat depots if the food supply furnishes much of the acid (6). Levene and Rolf (7) have shown that in liver lecithin linolenic acid exceeds linoleic acid. However, Turner (8) found no linolenic acid in sheep liver.

Fatty Acids in Nutrition. III

Pure methyl linolenate was prepared from linseed oil by the method of Rollett (9). The hexabromide was recrystallized until it melted at 180–181° (uncorrected). This assured the elimination of all but traces of other fatty acids and their bromides. The hexabromide was then debrominated, esterified, and distilled at less than 1 mm. The water-clear ester was stored *in vacuo* until used. Special precautions were taken to prevent oxidation after each sealed tube was opened. The methyl linolenate was fed at a low level only. Results with three rats are given in Chart
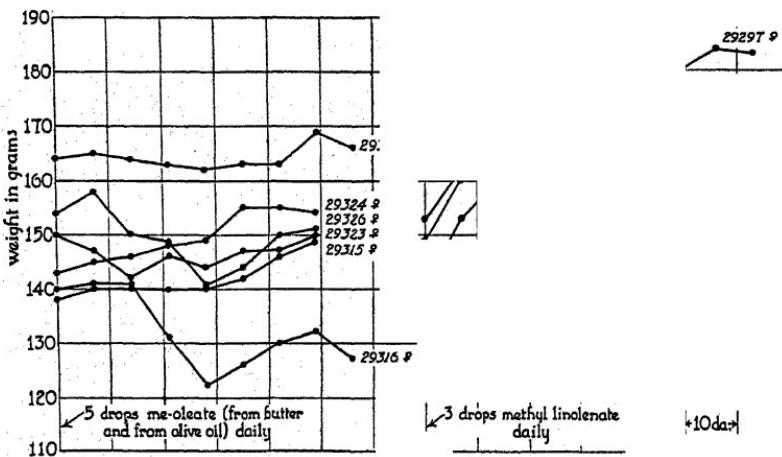
CHART II. Weight curves showing the growth response of rats to methyl oleate and methyl linolenate. Rats receiving methyl oleate show no renewed growth or clearing of the skin. Methyl linolenate quickly cleared the skin and the growth rate equaled that due to a like quantity of methyl linolate.

II. The gain in weight is almost identical with that for 3 drops of methyl linolate and the skin clears with great rapidity. It seems that linolenic acid can replace linoleic acid completely in the curing of rats suffering from a deficiency of fat.

α-Eleostearic Acid—Tung (China wood) oil is not ordinarily considered edible, but it has no harmful effects on rats when fed in small quantities. It is composed largely of the glyceride of α-eleostearic acid. A small amount of other unsaturated acids is present but linoleic and linolenic acids have not been reported present. α-Eleostearic acid melts at 48° and may be readily

changed into the β acid which melts at 71°. Since they readily absorb only 2 molecules of bromine the eleostearic acids were formerly considered isomeric with linoleic acid. The recent work of Böeseken and coworkers (10) shows that there are three double bonds in the eleostearic acids and that they are isomeric with linolenic acid. α-Eleostearic acid and its glycerides absorb oxygen very rapidly from the air and it is of interest to know whether this
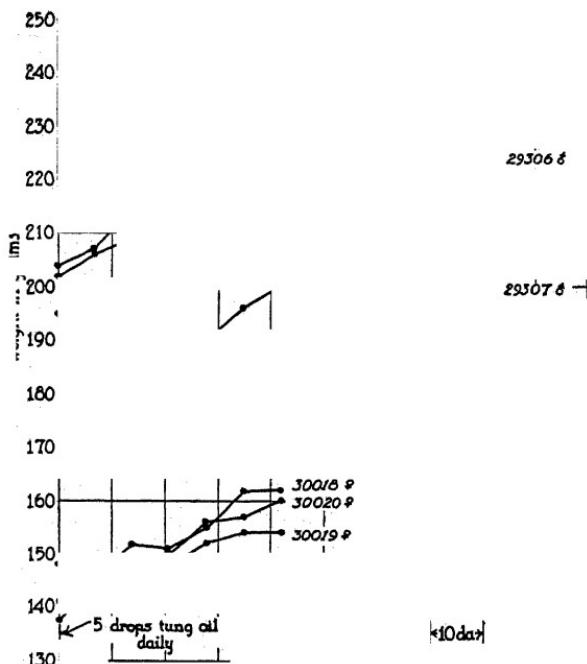
CHART III. Weight curves showing the slow response of rats to tung oil. The skin cleared very slowly.

acid which does not occur in animals can replace the more common linolenic acid. Tung oil was first fed to six rats. Slow but positive cures were effected. The skin gradually improved. The very gradual growth (Chart III) indicated that a trace of impurity rather than α-eleostearic acid was causing the response.

Pure α-eleostearic acid, m.p. 44–45°, was prepared from Florida tung oil.[1] This was given to rats in 5 drops doses for 2 weeks, but

[1] This oil was kindly furnished by Dr. J. S. Long, Lehigh University. It was the 1928 crop and had been kept under nitrogen.

they did not eat it well and the experiment was of little value. No tendency toward improvement was seen (Chart IV).

Methyl-α-eleostearate was then prepared by two methods. According to the first the acid was dissolved in an equal volume of absolute methyl alcohol and enough methyl alcoholic hydrogen chloride added to make a 4 per cent solution of HCl. This was left under CO_2 at room temperature overnight. The ester was purified by washing with dilute Na_2CO_3, distilled water, and $CaCl_2$ brine. The ester was finally taken up in ether, dried over anhydrous Na_2SO_4, and finally recovered *in vacuo*.
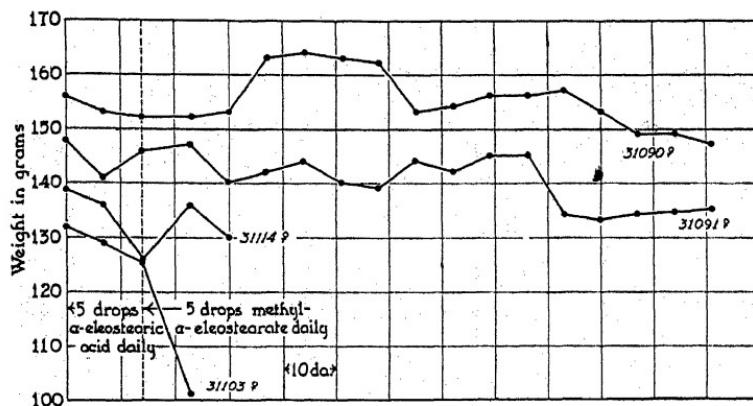
CHART IV. Weight curves showing the response of rats to α-eleostearic acid and to methyl-α-eleostearate. This acid fails to improve the skin or increase the weight.

This preparation is of high quality without distillation. Distillation must be avoided since it causes a rearrangement into the isomeric form, methyl-β-eleostearate. The α-eleostearate was sealed *in vacuo* and fed by dropping from a syringe so that the air was always excluded.

The other preparation was made by the very mild reagent, diazomethane.[2] The reaction goes smoothly and completely to give a high quality product requiring little or no purification.

Since there is some confusion in the literature concerning the

[2] We are indebted to Professor Lee I. Smith of the Department of Organic Chemistry for the first preparation made and for the detailed technique used by us.

rearrangement of α-eleostearic acid into β-eleostearic acid we checked our esters by saponifying small samples and recovering the free acid. After a single crystallization from alcohol the melting point was always between 43–45°. But when the methyl ester was distilled at 5 mm. pressure and the distillate was saponified, the β acid was recovered. After a single crystallization from alcohol it melted at 67–68°.
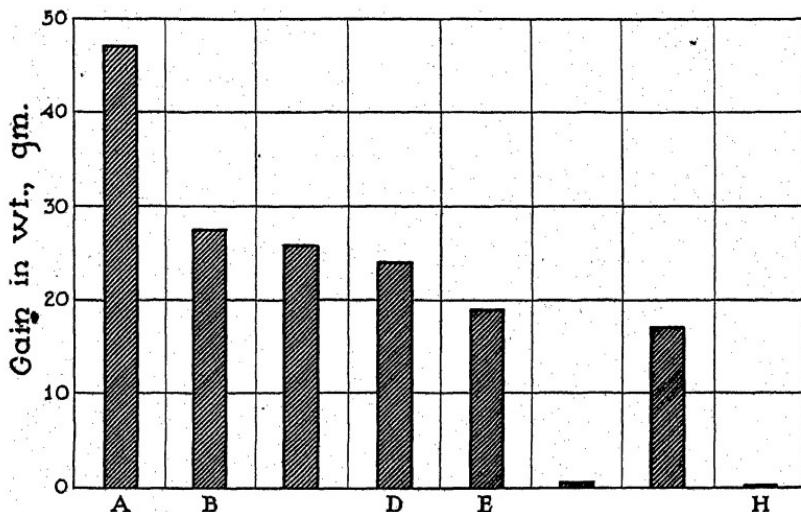
CHART V. A summary of the results given in the previous charts. The columns give the average maximum gains in weight of groups receiving the supplements over a period of 40 days. Column A, $7\frac{1}{2}$ drops of methyl linolate daily; Column B, 3 drops of methyl linolate daily; Column C, 3 drops of methyl linolenate daily; Column D, 3 drops of methyl linolate plus methyl linolenate (1:1 mixture) daily; Column E, 3 drops of methyl linolate plus methyl linolenate (1:1 mixture) plus 10 per cent methyl arachidonate daily; Column F, 5 drops of methyl oleate (from butter and from olive oil) daily; Column G, 5 drops of tung oil (90 per cent eleostearic acid) daily; Column H, 5 drops of methyl-α-eleostearate daily.

All of the preparations gave the same nutritional results. Some curves are shown in Chart IV. There is no evidence of improvement, either in weight or in skin condition.

DISCUSSION

A general summation of the comparisons of oleic, linoleic, linolenic, and α-eleostearic acids is given in Chart V. In all cases

where considerable growth took place the skin cleared and the rats
were generally improved.   A better muscle tone is always notice-
able after a rat has been cured.

By this work oleic acid has been definitely grouped with the
saturated acids as ineffective in the curing of rats subnormal
because of the lack of fat.   This substantiates the arguments put
forth in the second paper of this series (2) that it is possible for
animals to synthesize from carbohydrates large amounts of fat
and still suffer from a fat deficiency.   The review of the literature
will not be repeated here but it seems clear that warm blooded
animals synthesize only the saturated acids and oleic acid and that
they are dependent upon the food supply for linoleic and linolenic
acids.   One of these two acids must be ingested by the rat if it is
to survive and our findings indicate that they are interchangeable
in the tissues.   Further work is being done on the relative values
of the two.

The comparison of whole tung oil with methyl-α-eleostearate is
interesting.   Since the α-eleostearic acid does not have any cura-
tive effect it is evident that there is an acid in tung oil in small
amounts which causes the renewed growth.   Similar effects were
seen when 15 drops of butter were fed daily to rats (2).   Since pure
oleic acid and the saturated fatty acids are ineffective, small
amounts of undetermined acids are assumed to be present.   These
acids are probably linoleic or linolenic.

A mixture of linoleic and linolenic esters is of no more value
than either of the esters alone (Chart V, Column D).   This is
interesting since tissues normally have a mixture of the two.   When
methyl arachidonate was added as 10 per cent of the mixture the
animals uniformly showed less response (Chart V, Column E).   The
reason for this is not at all clear.   Lard contains appreciable
amounts of arachidonic acid and it is one of the best curative fats.
Liver and liver fat are rich sources of arachidonic acid.   Both have
been used by us as preventives for the fat deficiency and have
proved highly effective.   Since there is no reason to attribute toxic
effects to small amounts of arachidonic acid it seems probable that
some of the purified arachidonic acid which we have fed has been
altered in the process of preparation.

CONCLUSIONS

1. Both linolenic acid and linoleic acid are effective in curing rats suffering from a fat deficiency. They seem to be about equal in value and can replace each other in the tissues.

2. Oleic acid is ineffective in the curing of sick rats and is classed with the saturated acids.

3. α-Eleostearic acid, an isomer of linolenic acid, is ineffective in curing sick rats. This might be attributed to its high melting point.

4. Tung oil, like butter, has enough undetermined unsaturated acids to effect slow cures.

5. Mixtures of linoleic and linolenic esters are no more effective than a single ester, while the addition of a preparation of methyl arachidonate has a slight unexplained depressing effect.

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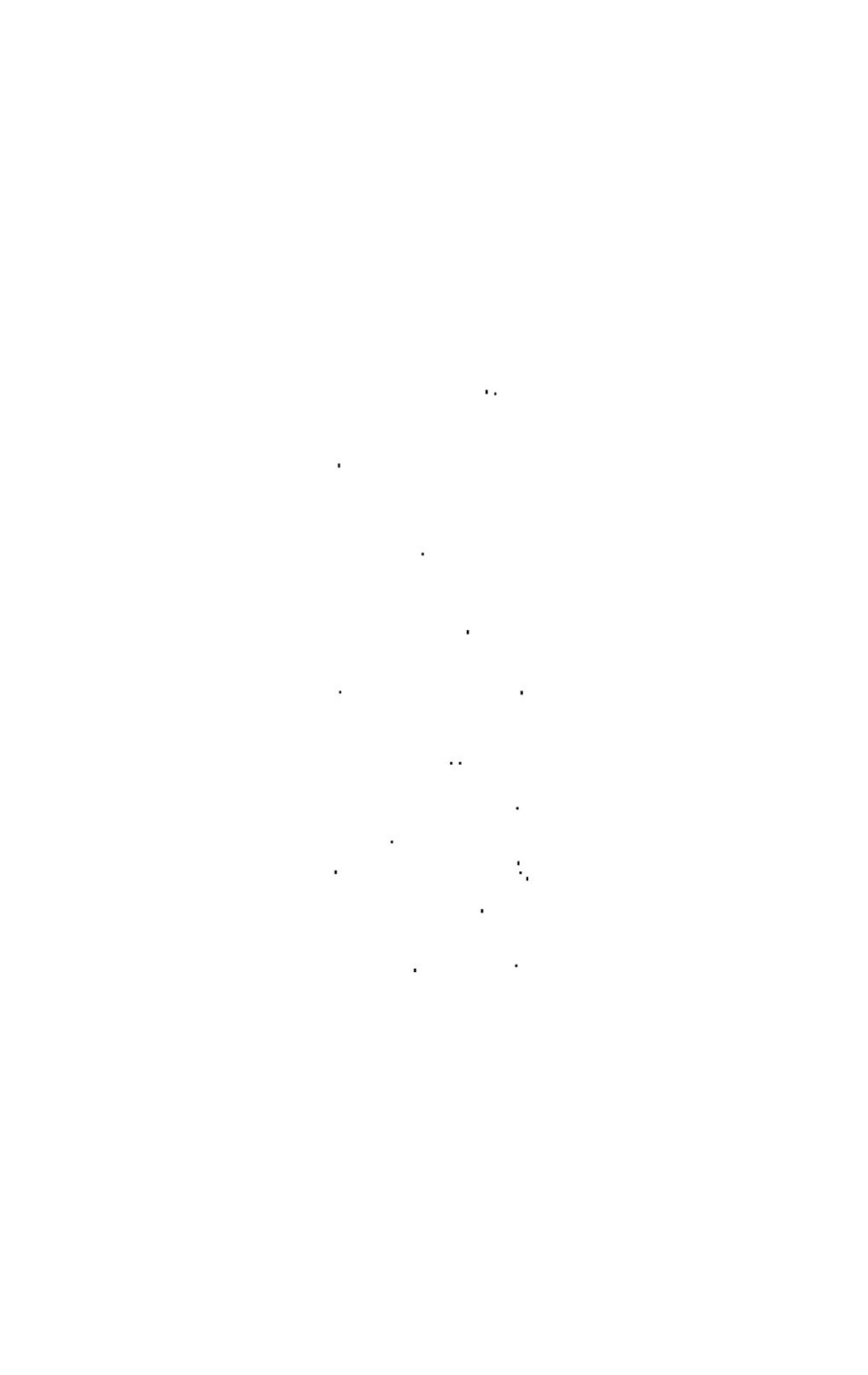
THE EFFECT OF INSULIN ON THE PHOSPHORUS COMPOUNDS OF MUSCLE*

By STANLEY E. KERR and M. ELEANOR BLISH

(From the Department of Biological Chemistry, American University of Beirut, Beirut, Syria)

The discovery that insulin causes a decrease in the inorganic phosphate of both blood and urine (1) attracted a number of investigators (1–4) to study the effect of insulin on the phosphorus compounds of muscle. Incomplete knowledge of the nature of muscle phosphorus and the use of a method (5) for determining "lactacidogen," which later proved to be incorrect (6), led to conflicting results. The discovery of phosphocreatine and adenosine triphosphate in muscle, and the publication of a new method for determining hexosephosphate (7) suggested to us the need for reinvestigating the effect of insulin on the distribution of phosphorus in muscle. While we were engaged in this study, the publications of Cori and Cori (8) appeared, showing that insulin causes the hexosemonophosphate content of muscle to increase, the effect being due primarily to increased epinephrine output. Our studies were continued, since they dealt with the distribution of a number of phosphorus compounds. With the method of Embden and Jost (7) for determining the hexosemonophosphate, and with rabbits instead of rats, our experiments confirm the results of Cori and Cori in showing that insulin causes an increase of hexosemonophosphate in muscle. This change we find to be accompanied by a decrease in the sum of the inorganic phosphorus and phosphocreatine.

EXPERIMENTAL

In order to measure the effect of insulin, the normal muscle was first removed under amytal anesthesia, insulin was injected, and after an interval the corresponding muscle from the other leg was

removed. This method has the disadvantage of complicating the effects of insulin with those of anesthesia and the surgical procedure. Control experiments were therefore conducted to determine the effect of anesthesia and the removal of one muscle on the composition of the second, no insulin being given. Rabbits were chosen for the experiments in order to secure muscles large enough for determining most of the phosphorus compounds simultaneously.

A detailed description of a typical experiment will illustrate the general procedure followed. A rabbit, fasted overnight, was anesthetized by intravenous injection of amytal freshly prepared according to the method described by the manufacturer (Lilly). The dosage was 60 to 80 mg. per kilo. Amytal was chosen as the most suitable anesthetic because of its relatively slight effect on carbohydrate metabolism (9) and its prevention of insulin convulsions, thus eliminating the possible effects of muscle exercise. As soon as anesthesia was complete, the calf muscles (gastrocnemius, plantaris, and soleus) of one leg were quickly removed in one group after tying off the muscles as close to the origin as possible, in order to prevent subsequent hemorrhage. The muscle was immediately plunged into liquid air. The incision was then closed, a blood specimen was taken by heart puncture, insulin was injected subcutaneously or intraperitoneally, and the animal was surrounded by cloths to keep it warm. The rabbit remained perfectly quiet for the next 3 hours, although consciousness returned after an hour. At the end of the insulin period the animal was again anesthetized with amytal or ether, the muscles of the other leg were removed and frozen in liquid air, and a second specimen of blood was taken. Many animals were lost when the second dose of amytal was given, respiration ceasing suddenly when sufficient amytal was used to permit removal of the muscle without pain. Hence, in the later experiments ether was given for the removal of the second muscle, the operation being performed as rapidly as possible to avoid the possible effects of ether on muscle composition.

The two muscles were next prepared for analysis. After a preliminary crushing to coarse lumps, the frozen muscle was ground to a fine powder in a large porcelain mortar previously chilled to a very low temperature by successive portions of liquid air. The powdered muscle was transferred to previously weighed

glass-stoppered Erlenmeyer flasks containing measured quantities of 5 per cent trichloroacetic acid. One small flask containing 20 cc. of acid received approximately 2 gm. of the frozen powdered muscle, this specimen being used for determining the phospho-creatine and inorganic phosphorus. It was weighed immediately after the addition of the muscle, shaken for a few minutes, then filtered through a chilled funnel. The determination of inorganic phosphorus and phosphocreatine was begun at once. The second flask, intended to receive the larger specimen of muscle, contained 40 cc. of 5 per cent trichloroacetic acid. After addition of the frozen muscle it was allowed to come to room temperature, then weighed, and an additional quantity of 5 per cent trichloroacetic acid added to make the final dilution 1 gm. of muscle plus 9 cc. of acid. This was considered a 1:10 dilution. After repeated shaking the specimen was filtered, usually at the end of an hour. The filtrate was used for determinations of pyrophosphate, total acid-soluble phosphorus, hexosemonophosphate, and in a few cases total purine.

Analytical Methods

Immediately after securing the protein-free trichloroacetic acid filtrate, the true inorganic phosphorus was separated from phos-phocreatine by the calcium precipitation method of Fiske and Subbarow (10), and the determination of each made in triplicate. In the experiments where phosphocreatine was not determined, the sum of phosphocreatine and inorganic phosphorus was de-termined by the method of the same authors.

The filtrate from the larger muscle sample was used for all other determinations. Total acid-soluble phosphorus was determined in triplicate by the method of Fiske and Subbarow (11). The de-termination of pyrophosphate was made by subtracting the sum of the inorganic and phosphocreatine phosphorus from the total inorganic phosphorus present after 7 minutes hydrolysis in normal hydrochloric acid at 100° (Lohmann (6)). 1 cc. portions of the protein-free filtrate were mixed with 1 cc. of 2 N hydrochloric acid in test-tubes, the tubes were covered with tin-foil and immersed in boiling water in a covered container for 7 minutes, and then placed in cold water after removal from the bath. The hydrolysis of the filtrate from the normal and insulinized muscles was carried

out simultaneously in order to secure perfectly comparable conditions. The inorganic phosphorus liberated during the hydrolysis was determined colorimetrically on the entire set of tubes together; all were compared against one standard. The general method of Fiske and Subbarow (11) was adopted, their Molybdate II and a dilution of 25 cc. being used. Hexosemonophosphate was determined by the method of Embden and Jost (7) in duplicate or triplicate. The figures obtained by this method actually represent the hexose bound to phosphate, but we have calculated the phosphorus content on the assumption that only the monophosphoric ester is present. That this assumption is correct is indicated by the data of Cori and Cori (8).

The blood specimens were analyzed for glucose by Benedict's 1928 method (12). The water content of muscle was determined in two experiments by drying weighed specimens of the powdered muscle at 110° for 24 hours.

<center>DISCUSSION</center>

A comparison of the normal and insulinized muscle of the same animal is presented in Table I. In the twenty-two experiments all but two of the rabbits were anesthetized with amytal. In Experiments 29 and 30 ether and morphine were used. Table II presents the results of the control experiments, in which the same procedure was used except that no insulin was administered.

The analyses indicate that the sum of inorganic phosphate and phosphocreatine is definitely decreased as the result of insulin, an increase occurring in only three out of the twenty-two experiments, while in the control experiments the changes are irregular. Although the changes in phosphocreatine are not entirely uniform, most of the experiments indicate that insulin causes this substance to decrease in muscle, whereas the opposite occurs in the control experiments. The changes in inorganic phosphate are irregular in both control and insulin experiments.

The decrease in the sum of phosphocreatine and inorganic phosphorus is sufficient to explain the attraction of inorganic phosphorus from the blood to muscle. However, the actual relationship of phosphocreatine and inorganic phosphorus in the living muscle is certainly disturbed to a considerable extent by the contraction caused by immersion in liquid air. During the time

required for complete freezing of the calf muscles of a rabbit this contraction may cause the breakdown of a significant quantity of phosphocreatine with a corresponding increase of inorganic phosphorus. The decrease in the sum of inorganic and phosphocreatine phosphorus is probably more representative of the true state of affairs than the individual values for these two substances. The lowering of the phosphocreatine content of muscle observed by us confirms similar findings by Mackler and Olmstead (13) in experiments on frogs. These authors found also an increase in the quantity of true inorganic phosphorus.

The amount of inorganic phosphate found after hydrolyzing the muscle filtrate for 7 minutes in normal hydrochloric acid (representing the true inorganic phosphate, phosphocreatine, and pyrophosphate) is definitely reduced by insulin as compared with the control experiments. This is accounted for chiefly by the decrease in the sum of phosphocreatine and inorganic phosphate, but in certain experiments it is due partly to a lowering of the pyrophosphate. The changes in pyrophosphate are too poorly defined to attribute them to insulin.

No uniform change in the total acid-soluble phosphorus is observable, the average remaining practically unchanged.

After subtracting the inorganic phosphorus found after 7 minutes hydrolysis from the total acid-soluble phosphorus, the remaining organic phosphorus is found to be higher in the insulinized muscle than in the normal in seventeen of the twenty-two experiments, but a similar increase is found in the control experiments where no insulin was given. This organic phosphorus is composed chiefly of hexosemonophosphate and adenylic acid. Determination of the former showed a marked increase after insulin, the average increase for fifteen experiments amounting to 43 per cent of the initial value. In seven control experiments a smaller increase occurred, the average rise being 16 per cent of the normal value. The increase of hexosemonophosphate observed in our experiments confirms the results of Cori and Cori (8). Their findings indicate that the rise of hexosephosphate is not a direct effect of insulin, but is due to an increased epinephrine output brought about by the hypoglycemia, since insulin produces no increase of the ester in adrenalectomized animals, nor when glucose is given simultaneously to prevent hypoglycemia.

TABLE I

Comparison of Normal and Insulinized Muscles of Same Rabbit

Experiment No.	Muscle sample	Insulin dosage (units per kg.)	Interval between insulin and removal of muscle (min.)	Blood sugar (mg. per 100 cc.)	Blood inorganic P (mg. per 100 cc.)	H₂O in muscle (per cent)	Phosphocreatine (1) (mg.)	Inorganic P (2) (mg.)	Sum of phosphocreatine and inorganic P (1)+(2) (mg.)	Inorganic F after 7 min. hydrolysis (3) (mg.)	Pyrophosphate P (3)−((1)+(2)) (mg.)	Total acid-soluble P (4) (mg.)	Organic P (4)−(3) (mg.)	Hexosemonophosphate (5) (mg.)	Undetermined P (4)−(3)−(5) (mg.)	Remarks
6	Normal	16	200	71			38.2	22.2	60.4	106.4	46.0	149.8	43.4			Amytal anesthesia
	After insulin						53.3	22.8	76.1	122.6	46.5	167.3	44.7			"
7	Normal	20	215	66			51.4	23.6	75.0	120.3	45.3	167.4	47.1			"
	After insulin						46.7	16.9	63.6	119.1	55.5	160.0	40.9			"
9	Normal	30+	185	63			53.0	28.4	81.4	123.1	41.7	170.7	47.6			"
	After insulin	7		128			52.1	22.9	75.0	110.1	35.1	167.7	57.6			"
11	Normal	30	210	50			37.2	28.3	65.5	115.0	49.5	158.0	43.0			"
	After insulin			108			35.4	28.2	63.6	105.4	41.8	163.2	57.8			"
13	Normal	43	195	46			50.9	33.1	84.0	130.0	46.0	169.0	39.0			"
	After insulin			114			32.3	35.9	68.2	106.4	38.2	170.4	64.0			"
20	Normal	20+	180	49	4.5	76.5	47.8	22.9	70.7	122.2	51.5	173.3	51.1			"
	After insulin	20		102	5.3	76.7	44.5	33.2	77.7	118.0	40.3	170.6	52.6			"
21	Normal	20+	165	65	2.9	76.5	52.5	23.8	76.3	126.0	49.7	171.2	45.2			"
	After insulin	20		105	2.9	75.8	46.1	30.7	76.8	118.9	42.1	172.4	53.5			"
26	Normal	30+	180	53	2.9					120.8		164.1	43.3	17.0	26.3	"
	After insulin	15		142	2.8					114.2		163.0	48.8	22.0	26.8	"
27	Normal	30+	180	55						123.1		162.7	39.6	15.5	24.1	"
	After insulin	15			2.7					121.4		161.0	39.6	17.7	21.9	"

Exp. No.	Condition												Notes
28	Normal	30+	172	93	3.1		123.5		162.9	39.4	7.5	31.9	"
	After insulin	15		60	2.8		121.2		169.9	48.7	10.5	38.2	
29	Normal	20	90	171	5.1	87.4	121.4	34.0	163.1	41.7	5.7	36.0	Both muscles under ether and morphine
	After insulin			76	5.5	76.8	109.8	33.0	157.2	47.4	14.4	33.0	
30	Normal	20	90	172	5.5	84.9	121.1	36.2	166.2	45.1	9.6	35.5	"
	After insulin			68	4.7	81.4	116.5	35.1	157.9	41.4	12.0	29.4	
66	Normal	40	185	76	4.2	84.4	121.2	36.8	164.7	43.5	20.6	22.9	Amytal. Ether given at time of removal of cle
	After insulin			54	3.0	85.7	124.6	38.9	174.8	50.2	25.2	25.0	
67	Normal	40	180	140		93.2	126.0	32.8	172.3	46.3	22.2	24.1	"
	After insulin			36		82.1	124.6	42.5	176.1	51.5	27.5	24.0	
72	Normal	40	130	82	3.5	91.8	127.0	35.2	168.2	41.2	19.4	21.8	"
	After insulin			91	3.0	81.0	120.7	39.7	168.9	48.2	25.4	22.8	
73	Normal	40	120	91	4.3	93.6	129.4	35.8	176.7	47.3	22.2	25.1	"
	After insulin			36	3.6	87.7	128.0	40.3	177.8	49.8	25.4	24.4	
75	Normal	30	180	123		93.5	127.2	33.7	177.2	50.0	13.1	36.9	"
	After insulin			97		78.9	117.0	38.1	172.4	55.4	21.2	34.2	
76	Normal	30	150	111	2.7	93.9	130.7	36.8	184.6	53.9	10.8	43.1	"
	After insulin			12	2.4	72.9	118.9	46.0	172.9	54.0	19.0	35.0	
77	Normal	30	185	133	3.2	99.2	133.8	34.6	176.8	43.0	6.0	37.0	"
	After insulin			143	2.9	91.5	132.7	41.2	174.8	42.1	19.0	23.1	
78	Normal	30	190	111		89.0	123.5	34.5	176.1	52.6	12.6	40.0	"
	After insulin			24		78.1	116.8	38.7	164.8	48.0	21.2	26.8	
79	Normal	30	175	156	3.6	99.1	135.1	36.0	174.7	39.6	9.8	29.8	"
	After insulin			75	2.7	85.6	124.8	39.1	171.4	46.6	15.4	31.2	
80	Normal	30	180	156	3.0	96.2	135.7	39.5	183.5	47.8	12.6	35.2	"
	After insulin			33	2.4	83.1	126.2	43.1	168.8	42.6	16.7	25.9	
Average of 22 experiments	Normal			122	3.7	85.2	124.7	39.8	169.7	45.0	13.6	31.3	
	After insulin			60	3.3	78.2	119.0	40.8	168.3	49.3	19.5	28.1	

TABLE II

Control Experiments. Effect of Amytal and Removal of Muscles of One Leg on Composition of Muscles of Other Leg

Experiment No.	Muscle sample	Interval between removal of two muscles (min.)	Blood sugar (mg. per 100 cc.)	Blood inorganic P (mg. per 100 cc.)	P per 100 gm. muscle									Remarks
					Phosphocreatine (1) mg.	Inorganic P (2) mg.	Sum of phosphocreatine and inorganic P (1)+(2) mg.	Inorganic F after 7 min. hydrolysis (3) mg.	Pyrophosphate P (3)−((1)+(2)) mg.	Total acid-soluble P (4) mg.	Organic P (4)−(3) mg.	Hexosemono-phosphate (5) mg.	Undetermined P (4)−(3)−(5) mg.	
8	First	185	158	4.2	53.4	24.1	77.5	123.7	46.2	168.1	44.4			Both muscles removed under amytal
	Second				44.0	28.5	72.5	115.6	43.1	161.1	45.5			
12	First	175	412	4.1	41.9	24.5	66.4	112.6	46.2	167.7	55.1			"
	Second				38.6	30.7	69.3	113.0	43.7	169.5	56.5			
14	First	185	124	4.3	42.0	28.3	70.3	120.7	50.4	168.5	47.8			"
	Second		184		44.4	26.7	71.1	111.7	40.6	168.3	56.5			
61	First	180	100	4.0	46.2	35.0	81.2	118.3	37.1	168.9	50.6	20.6	30.0	"
	Second		154		50.2	31.5	81.7	121.2	39.5	175.3	54.1	21.2	32.9	
64	First	185	106	4.3	65.8	28.8	94.6	142.0	47.4	181.8	39.8	11.3	28.5	"
	Second		111		66.3	31.9	97.2	141.0	43.8	179.9	38.9	10.1	28.8	
56	First	195	82	4.1	57.2	30.3	87.2	136.0	48.5	178.6	42.6	9.6	33.0	First muscle removed under amytal, ether given at time of removal of second muscle
	Second		111		58.8	43.4	102.2	144.0	41.8	196.0	52.0	9.6	42.4	

57	180	First	144	3.7	55.2	31.4	86.6	128.0	41.6	169.9	41.7	10.8	30.9	"	"
		Second	212	2.9	52.3	27.7	80.0	129.1	49.1	181.5	52.4	15.3	37.1		
59	170	First	94	3.9	56.1	30.2	86.3	127.0	40.7	165.3	38.3	11.3	27.0	"	"
		Second	160	3.4	60.4	22.7	83.1	125.6	42.5	170.0	44.4	12.7	31.7		
60	180	First	112	5.0	49.7	32.0	81.7	125.0	43.3	163.2	38.2	14.7	23.5	"	"
		Second	117	5.0	53.0	34.1	87.8	128.4	41.3	170.3	41.9	17.5	24.4		
62	180	First	95	5.2	58.8	32.7	91.5	131.8	40.3	174.2	42.4	14.2	28.2	"	"
		Second	138	4.9	60.3	34.4	94.7	130.7	36.0	184.0	53.3	21.0	32.3		
Average	182	First	107	4.4	52.6	29.7	82.4	126.5	44.2	170.6	44.1	13.2	28.7		
		Second	176	4.1	52.8	31.1	84.0	126.0	42.1	175.6	49.6	15.3	32.8		

Many of our initial values for hexosephosphate are higher for resting muscle than the figures obtained by Cori and Cori for rat muscle. These authors state that it is difficult to secure resting values for rabbit muscle. This is perhaps due to the impossibility of cooling quickly as large a mass of muscle as the gastrocnemius of a rabbit by the method these authors used and also to the contraction which results when liquid air is used, as in our experiments. However, the desirability of performing a series of analyses on the same muscle necessitated the choice of the larger animal. On comparing our results with those of Cori and Cori, it is evident that rat and rabbit muscle differ considerably in their total acid-soluble phosphorus content, due apparently to a smaller amount of phosphocreatine and inorganic phosphorus in the muscle of the rabbit as compared to that of the rat.

The remaining fraction of the organic phosphorus (recorded in our tables as "undetermined" phosphorus) was determined by difference, by subtracting the phosphorus attached to hexose from the "organic" phosphorus.

In the control experiments the undetermined phosphorus (chiefly adenylic acid) increases due to the amytal or to the operative procedure. After insulin a decrease occurs in about half the experiments, the average for fifteen animals showing a drop of about 11 per cent. The inference would appear to be that insulin causes a lowering of the nucleotide or of the unrecognized phosphorus compound remaining. However, the errors of three determinations accumulate in the value for undetermined phosphorus, hence this inference should be regarded with suspicion. A rough approximation of the amount of adenylic acid in muscles was made in three experiments by determining the total purine in the muscle filtrates. Since very little nucleoside and free purine are found in muscle (14), the phosphorus equivalent was calculated, assuming all purine to be adenylic acid. No significant change was observed after insulin.

The changes characteristic of insulin, therefore, are the increase of hexosemonophosphate and the decrease in the sum of phosphocreatine and inorganic phosphate.

In order to study the effects of insulin uncomplicated by anesthesia and the shock caused by removing the normal muscle, an attempt was made to compare the muscles of normal control

animals with those treated with insulin. This was the method adopted by Cori and Cori (8), as we later learned. The insulinized animals were anesthetized and the muscles removed only at the end of the insulin period. The large individual differences found in the muscle composition of the normal rabbits, however, made such a comparison hopeless. The values for various constituents in the muscles of twenty-six normal rabbits covered the entire range of sixteen insulinized animals. In this group of experiments the muscles were removed from some animals under amytal anesthesia, from some under ether, and from still others stunned by a blow on the head. The conditions of removal affect the muscle composition sufficiently to render even averages for this group of experiments of doubtful value; hence, we believe it safer to judge the effect of insulin from the experiments in which both muscles were removed from the same animal under amytal anesthesia.

In interpreting results it should be borne in mind that considerable differences may be found in the composition of the right and left muscles removed simultaneously from the same animal.

The phosphorus required for the synthesis of the hexose ester may apparently have phosphocreatine as its source. In the experiments where the total acid-soluble phosphorus after insulin remained unchanged or decreased, the increased quantity of phosphate attached to hexose must have been derived either from the phosphocreatine or from the inorganic phosphate of the muscle. The apparent decrease of total acid-soluble phosphorus after insulin in some experiments, occurring simultaneously with a decrease in the blood phosphate, may possibly be explained by an unequal distribution of phosphorus in the muscles of the body. Analyses of the right and left muscles removed at the same time show similar variations. Differences in the water content of muscle before and after insulin are not sufficient to account for the large differences found in the total phosphorus in certain experiments, judging by the results of water determinations in two experiments.

SUMMARY AND CONCLUSIONS

1. A comparison is made of the content of phosphocreatine, inorganic phosphorus, pyrophosphate, hexosemonophosphate, and

residual phosphorus in the skeletal muscle of the rabbit before and after insulin dosage.

2. After allowance is made for the effects of amytal anesthesia and the operative procedure, the results of the insulin are found to be a decrease in the sum of phosphocreatine and inorganic phosphorus, and an increase in hexosemonophosphate.

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THE DIURNAL VARIATIONS OF THE CHOLESTEROL CONTENT OF THE BLOOD*

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The view is held by a large number of the more recent workers that the "chaotic state of the literature" on the subject of cholesterol metabolism, in particular, and of lipid metabolism, in general, is due almost entirely to the large number of inaccurate methods that have had common usage. On the other hand, from 1914 to date, Bloor and his associates have carefully investigated many aspects of lipid metabolism with standardized methods. By far the greater amount of the work on cholesterol published in the last 15 years is based upon methods which, for the main part, are strikingly similar. It is true that the number of extraction processes in use is legion, but it cannot be denied that many of these (see Myers and Wardell (1), Bloor, Pelkan, and Allen (2), Sackett (3), Leiboff (4), Forbes and Irving (5)) are adequate. It cannot be denied that the extremely variable results reported in the literature are due in part to the methods employed, but this does not explain the situation completely.

The same state of affairs apparently exists in studies of the physiological variations in blood cholesterol. Iscovesco (6) and Horiuchi (7) noted the constancy of the blood cholesterol in rabbits. This was more recently confirmed by Glusker (8), in dogs. McClure and Huntsinger (9) showed that, with few exceptions, only insignificant changes occur at hourly intervals for 7 hours in fasting normal young adults. Very recently, however, McEachern and Gilmour (10) studied the plasma cholesterol in

twenty-eight normal individuals for 5 consecutive hours in the fasting state and found large variations. They concluded that such variations render single or haphazard studies of blood cholesterol of doubtful value. It is interesting to note that McClure and Huntsinger (9) and McEachern and Gilmour (10) used exactly the same method for the determination of plasma cholesterol (Bloor, Pelkan, and Allen (2)).

Mirsky and Bruger (11) have shown in a study of the colorimetric estimation of cholesterol by the Liebermann-Burchard color reaction that temperature control is an extremely important factor in the production of the color. Variations of 15 per cent and higher in duplicate determinations of single blood specimens are extremely common unless the temperature of reaction is strictly controlled. It is conceivable that the wide variations observed in the cholesterol content of the blood in fasting normal individuals, as reported in the literature, may be due to this factor. Our own studies do not support the results obtained by McEachern and Gilmour (10); these writers report wider variations in the blood cholesterol in 5 hours than we have observed in 24 hours.

Material and Methods

The present report consists of two series of experiments, the first of which comprises a group of nine unselected patients in whom the whole blood cholesterol was determined every 2 hours for 24 hours (Table I). These patients were bedridden and were eating a standard diabetic diet as outlined in Table I. Breakfast was served between 7.30 and 8.00 a.m., lunch between 11.30 a.m. and 12.00 noon, and dinner between 4.50 and 5.30 p.m.

The second series comprises a group of nine subjects in whom the plasma cholesterol was determined every hour from 9.00 a.m. to 1.00 p.m. (Table II). These subjects were in the postabsorptive state, no food or drink having been taken since dinner the evening preceding the test. Owing to unforeseen circumstances, two of the individuals were studied for only 3 and 2 hours respectively (Table II, Cases 14 and 18). Five subjects were normal; four were patients chosen from the wards, but the conditions of the experiment were the same.

In the first series, with the single exception of Case 8 (Table I), the whole blood cholesterol was determined by the method of

Diurnal Variations of Whole Blood Cholesterol in Human Subjects (Absorptive Group)

Case No. and sex	Diagnosis	Age	Whole blood cholesterol (mg. per 100 cc.)													24 hr. variations		8 a.m. to 12 m. variations	
			8 a.m.	10 a.m.	12 m.	2 p.m.	4 p.m.	6 p.m.	8 p.m.	10 p.m.	12 p.m.	2 a.m.	4 a.m.	6 a.m.	8 a.m.	Mean	Standard deviation	Mean	Standard deviation
																	per cent		per cent
1, F.	Diabetes mellitus, chronic nephritis, arteriosclerosis	57	324	369	347	275	342	329	358	329	363	333	363	382		343	±7.9	347	±5.3
2, M.	Arteriosclerosis, hypertension	54	196	195	186	184	205	179	184	166	116	183	170	166	186	178	±11.8	192	±2.3
3, "	Chronic arthritis	24		119	115	118	115	124		125	134	109	97		124	118	±8.1	117	±1.7
4, F.	Arteriosclerosis	60	166	163	173	179	165		133	155	149	142	158			158	±8.4	167	±2.5
5, "	Hypertension, osteoarthritis	50	176	176	179	173	166		161	160	158		158	142	161	165	±6.3	177	±0.8
6, M.	Diabetes mellitus	35	145	145	147	159	156	153	157	150	152	141	133	131	131	146	±6.4	146	±0.7
7, "	Cardiac decompensation	48	111	114	115	122	127	121	117	116	116	109	109	103	115	115	±5.2	113	±1.5
8,* M.	Chronic nephritis	23	500	492	416	492	462	467	487	469	509	499	498	505		483	±5.2	469	±8.1
9, F.	"	45	296	242	255	243	293	253	179	189	212	296	264	222	161	245	±13.1	264	±8.7
Average																	±8.0		±3.5

Case 3, received a diet of 60 gm. of carbohydrate, 45 gm. of protein, 105 gm. of fat; 1407 calories. Cases 1, 4, 5, 8, and 9 received 80 gm. of carbohydrate, 60 gm. of protein, 140 gm. of fat; 1876 calories. Cases 2, 6, and 7 received 100 gm. of carbohydrate, 75 gm. of protein, 175 gm. of fat; 2345 calories.

* Plasma cholesterol.

Myers and Wardell.[1] The plasma cholesterol of Case 8 (Table I) and of Cases 10 to 18 inclusive (Table II) was determined by Sackett's modification of Bloor's method (3). In the latter series, duplicate and, at times, triplicate determinations were made on single blood specimens, the temperature control procedure suggested by Mirsky and Bruger (11) being utilized.

TABLE II

Hourly Variations of Plasma Cholesterol in Human Subjects (Fasting Group)

Case No.	Diagnosis	Age	Sex	Plasma cholesterol (mg. per 100 cc.)					Summary of 9 a.m. to 1 p.m. variations	
				9 a.m.	10 a.m.	11 a.m.	12 m.	1 p.m.	Mean	Standard deviation
										per cent
10	Normal	28	M.	247	259	245	264	216	246	±6.8
11	"	25	"	227	203	221	227	240	223	±5.4
12	Diabetes mellitus	46	F.	189	195	195	188	187	191	±1.8
13	Mediastinal tumor	69	M.	348	343	373	328	369	352	±4.8
14	Normal	28	F.	178	182	172			177	(±2.3)
15	"	27	M.	191	194	206	205	206	200	±3.3
16	Peptic ulcer	45	"	174	167	167	162	159	166	±3.0
17	Normal	36	"	197	191	207	195	198	197	±2.7
18	Diabetes mellitus, arteriosclerosis	69	"	192	176				184	(±4.3)
Average...										±3.9

The figures in parentheses were not included in the average.

Results

Table I shows the degrees of variation that occur in the whole blood cholesterol in patients, with various diseases, determined every 2 hours for 24 hours in the absorptive state. The initial levels of blood cholesterol varied from low values (case of cardiac decompensation) to rather high figures (case of "nephrotic" glomerular nephritis). A study of our protocols shows that the higher the initial cholesterol level, the greater are the variations, but computations from our data disclose the interesting finding

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[1] We are indebted to Dr. J. A. Killian of the Department of Biochemistry for permission to use these data.

that the standard deviations were practically the same for all cases whether the mean cholesterol level was low, normal, or high.

The standard deviations were calculated from the formula, $\mu = \dfrac{\sqrt{\Sigma(d^2)}}{n}$, where $\Sigma(d^2)$ represents the summation of the squares of the individual deviations from the mean, and n the number of determinations. The standard deviation (μ) was then expressed in per cent on a basis of a mean of 100 mg. of cholesterol per 100 ml. of blood.

The whole blood cholesterol in each of the nine patients in this group varied by a standard deviation of ±8.0 per cent. The limits of variation were ±5.2 and ±13.1 per cent. The standard deviation for the morning hours (8.00 a.m. to 12.00 noon) was only ±3.5 per cent with limits of ±0.7 and ±8.7 per cent (Table I).

There was no apparent relationship between the type of clinical condition, the age and sex of the patient, and the degree of variation in the blood cholesterol. We shall be able to demonstrate later that normal subjects show the same degree of variation in the morning hours, and it may be safely stated here that the blood cholesterol of normal individuals undergoes physiological variations which are of the same magnitude as those found in pathological states. This statement must be modified only for those clinical conditions accompanied by severe cachexia or for patients *in extremis* from any cause, since in such instances, the cholesterol content of the blood is usually remarkably constant and is apparently unaffected by the administration of various substances which in all other cases produce definite effects on the blood cholesterol (to be published later).

Our protocols show clearly that the ingestion of food has no constant effect, either immediate or late, on the cholesterol content of the blood. In most cases, there was a small but gradual rise in the blood cholesterol in the early morning hours (4.00 a.m. to 8.00 a.m.).

Table II shows the degrees of variation that occur in the plasma cholesterol of five normal subjects and four patients, the determinations being carried out every hour from 9.00 a.m. to 1.00 p.m. in the fasting state (15 to 20 hours postabsorptive). The plasma cholesterol in each of the nine subjects varied by a stand-

ard deviation of ±3.9 per cent. The limits of variation were ±1.8 and ±6.8 per cent.

A comparison of these figures with those of the morning hours in the absorptive group (Table I) shows a striking similarity in the findings. They tend to demonstrate pertinently that the ingestion of food, or, at least, the type of food taken by our subjects, had little or no effect on the cholesterol content of the blood. As mentioned above, these results also demonstrate that normal subjects show practically the same degree of variation in the blood cholesterol as found in the pathological states.

DISCUSSION

The magnitude of the fluctuations in the cholesterol content of the blood found by Glusker (8) in dogs, and by McClure and Huntsinger (9) in human subjects is further supported by our own studies. Glusker found that the total cholesterol in each of ten dogs over a period from the 15th to the 23rd hour postabsorptive varied by a standard deviation of ±3.7 per cent. It is interesting to note that the blood cholesterol in each of our nine subjects over a period from the 15th to the 20th hour postabsorptive varied by a standard deviation of ±3.9 per cent.

We cannot explain the extremely wide variations in the blood cholesterol reported by McEachern and Gilmour (10) in twenty-eight normal fasting subjects. Calculations made from their data showed a standard deviation of ±9.5 per cent with limits of ±3.4 and ±16.5 per cent. These figures are considerably higher than those observed in our fasting subjects and in fact are also higher than those obtained in subjects studied every 2 hours for 24 hours in the absorptive state.

Since in all cases the variations in the blood cholesterol were considerably less in the morning hours than during any other part of the day (or night), it is suggested that cholesterol studies be carried out during the morning hours. Deviations of 1½ times or at most 2 times the standard deviation (i.e., ±5.8 to ±7.8 per cent) should be considered significant changes when any aspect of cholesterol metabolism is studied.

As regards the effect of food ingestion on the cholesterol content of the blood, the reports in the literature are more or less contradictory. In a very recent communication, Bloor (12) has

shown that the continuous feeding of diets, on the one hand high in fat, and on the other hand low in fat, produces definite changes in the level of plasma cholesterol of dogs and rabbits. The level of cholesterol is always higher on a high fat diet than on a low fat diet. He also demonstrated that the difference is not so great in the dog, but in the rabbit it is very marked. These observations reported by Bloor confirm the findings of a large number of workers who studied the effect of diet on the blood cholesterol in various species of animals. In man, however, the reports in the literature concerning the effect of food ingestion on the blood cholesterol are at variance. Muller (13) concludes that in man it is possible to obtain a small temporary rise in the blood cholesterol a few hours after a meal, which is missed in other species. Gardner and Gainsborough (14) believe that the variations in the plasma cholesterol in man could scarcely be due to direct absorption of cholesterol from the food into the blood plasma. Our own studies support the view of Gardner and Gainsborough. We observed no appreciable effect of food ingestion on the cholesterol content of the blood. The degree of variation was identical in both the absorptive and the fasting groups.

The part played by cholesterol in fat metabolism is still a moot question. The recent report of Bloor (12), referred to above, is of great significance. He concludes that cholesterol undoubtedly has other functions than that in fat metabolism. Recent work carried out in this laboratory concerning the effect of glucose ingestion, glucose and saline infusions, and adrenalin and insulin parenterally administered on the blood cholesterol (to be published later) supports the view, first propounded by Fishberg (15), that cholesterol may play a part in the equilibration of the osmotic pressure of the blood. The fluctuations observed in the cholesterol content of the blood may reflect such changes in the osmotic pressure incident to the metabolic processes continuously taking place in the animal organism.

SUMMARY AND CONCLUSIONS

The variations in the cholesterol content of the blood determined every 2 hours for 24 hours were studied in nine patients in the absorptive state. The whole blood cholesterol in each of these subjects varied by a standard deviation of ±8.0 per cent. The

standard deviation for the morning hours in this group was only ±3.5 per cent. In a fasting group, each of nine subjects studied every hour for 4 hours varied by a standard deviation of ±3.9 per cent.

The ingestion of food is shown to have no appreciable effect, either immediate or late, on the blood cholesterol.

The cholesterol content of the blood of normal individuals undergoes physiological variations which are of the same magnitude as those found in pathological states.

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ON THE INFLUENCE OF ETHYL ISOTHIOCYANATE, ETHYL THIOCYANATE, AND ALLYL ISOTHIO- CYANATE ON SULFUR METABOLISM IN RABBITS

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Plant materials containing mustard oils have been repeatedly investigated regarding their suitability as foodstuffs for domestic animals (1). The finding that the toxicity of rape- and mustard-cake is in part due to their content of mustard oil, led to a closer investigation of the physiological effects of these substances (2). Peterson (3) studied the metabolism of allyl isothiocyanate and allyl sulfide in pigs. He found that the addition of allyl isothio-cyanate and of allyl sulfide to the ration produced a gradual in-crease in the total sulfur excreted in the urine and a continuance of this high increase for about 10 days after the ingestion of the mustard oils had ceased, indicating that they were metabolized very slowly.

Our investigation on the influence of ethyl isothiocyanate, ethyl thiocyanate, and allyl isothiocyanate on the sulfur metabolism in rabbits was undertaken as part of a study of the fate of cyanides in the organism. It is known that one of the mechanisms which the body uses in the detoxication of cyanides is transformation into thiocyanates by combination of the CN group with labile sulfur. Richards and Wallace (4), who studied the influence of cyanides upon metabolism in dogs, noticed an increase in total nitrogen excretion, but state that the total sulfur excretion did not follow closely the excretion of total nitrogen. Smith and Malcolm (5) in their studies on the effects of cyanide poisoning, found an in-crease in total nitrogen after administration of cyanide, indicating an increased catabolism of body protein. But instead of a corresponding rise in the excretion of total urinary sulfur they

found in half of their cases a definite drop in the sulfur excretion. They explain this finding as probably due to the demand for sulfur in the detoxication of cyanides. We observed the same phenomenon in our rabbits on administration of either ethyl isothiocyanate, ethyl thiocyanate, or allyl isothiocyanate. In the case

TABLE I

Effect of Administration of Ethyl Isothiocyanate and of Ethyl Thiocyanate on Sulfur Metabolism

The figures are daily averages for 1 week.

	Date	Intake	Ethyl isothiocyanate				Ethyl thiocyanate			
			Total S excretion in urine	Retention	Total S excretion in per cent of intake	S retention in per cent of intake	Total S excretion in urine	Retention	Total S excretion in per cent of intake	S retention in per cent of intake
	1932	*mg. S*	*mg. S*	*mg. S*			*mg. S*	*mg. S*		
Control period	Jan. 15–21	128	71	57	56	44	77	51	60	40
" 22–28	128	72	56	56	44	79	49	62	38
Daily average..	Jan. 15–28	128	71	57	56	44	78	50	61	39
Ethyl isothiocyanate = 37 mg. S	Jan. 29–Feb. 4	165	78	87	47	53	72	93	44	56
	Feb. 5–11	165	76	89	46	54	83	82	50	50
Daily average..	Jan. 29–Feb. 11	165	77	88	46	54	77	88	47	53
Control period	Feb. 12–18	132	68	64	52	48	74	58	56	44
" 19–25	132	71	61	54	46	68	64	52	48
" 26–Mar. 3	132	71	61	54	46	79	53	60	40
	Mar. 4–10	132	73	59	55	45	75	57	57	43
Daily average..	Feb. 12–Mar. 10	132	71	61	54	46	74	58	56	44

of the ethyl compounds the total urinary sulfur excretion, calculated as per cent of intake, was markedly decreased in the experimental period, though the absolute values showed little variation, coming slowly back to normal in the after period. In the case of allyl isothiocyanate the excretion of total urinary sulfur calcu-

lated as per cent of intake, was also decreased during the experimental period, but rose perceptibly above that in the normal period after the administration of mustard oil had been stopped. No difference was found in the effects of ethyl isothiocyanate and ethyl thiocyanate.

The increased retention of sulfur during the ingestion of thiocyanates can hardly be explained by a lag in the excretion, as the

TABLE II

Effect of Allyl Isothiocyanate Administration on Sulfur Metabolism

The figures are daily averages for 1 week.

	Date	Sulfur				
		Intake	Total excretion in urine	Retention	Total excretion, per cent of intake	Retention, per cent of intake
	1931	*mg.*	*mg.*	*mg.*		
Control period.........	Oct. 23–29	164	115	49	70	30
" "	" 30–Nov. 5	164	123	41	75	25
" "	Nov. 6–12	164	106	62	65	35
Daily average.........	Oct. 23–Nov. 12	164	115	49	70	30
Allyl isothiocyanate = 13 mg. S..............	Nov. 13–19	180	108	72	60	40
" "	" 20–26	166	91	75	55	45
Daily average.........	Nov. 13–26	173	100	73	58	42
Control period.........	Nov. 27–Dec. 3	121	104	17	85	15
" "	Dec. 4–10	134	116	18	87	13
Daily average.........	Nov. 27–Dec. 10	128	110	18	86	14

percentage of ingested sulfur, which is excreted, is far below the normal. Since no combination with sulfur could occur in the handling of thiocyanates by the body, as it does in the detoxication of cyanides, other factors seem to be involved in the retention of sulfur during the ingestion of thiocyanates. In order to throw further light on this subject, experiments are being carried out on the mechanism of the interrelationship of cyanide and sulfur metabolism.

Adult male rabbits were used in all experiments. They were kept on a diet of alfalfa hay and rolled oats. After establishing the normal output of total urinary sulfur, three groups of rabbits were given an olive oil solution of ethyl isothiocyanate, ethyl thiocyanate, and allyl isothiocyanate, respectively, over a period of 2 weeks. Two rabbits received daily ethyl isothiocyanate, corresponding to 37 mg. of sulfur, two rabbits ethyl thiocyanate, corresponding to 37 mg. of sulfur, and three rabbits allyl isothiocyanate, corresponding to 13 mg. of sulfur.

Since Peterson (3) states that the increased sulfur excretion is confined mainly to the unoxidized part of the sulfur excreted, and that the curves for unoxidized sulfur and total sulfur rise and fall together, only the total urinary sulfur has been determined. Estimations were made according to the method of Benedict. The food was analyzed for sulfur according to the method of Neumann and Meinertz (6). In view of the uniformity of the results, a sample protocol is presented for each group (Tables I and II).

SUMMARY

The influence of ethyl isothiocyanate, ethyl thiocyanate, and allyl isothiocyanate on sulfur metabolism in rabbits has been studied.

In the case of the ethyl compounds the total urinary sulfur excretion, calculated as per cent of intake, was markedly decreased in the experimental period, coming slowly back to normal in the after period. No difference was found in the effects of ethyl isothiocyanate and ethyl thiocyanate.

In the case of allyl isothiocyanate the excretion of total urinary sulfur, calculated as per cent of intake, was also decreased during the experimental period, but rose perceptibly above that in the normal period after the administration of mustard oil had been stopped.

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THE INFLUENCE OF SUBSTITUENT GROUPS ON THE VISIBLE AND ULTRA-VIOLET ABSORPTION SPEC- TRA OF AMINO ACIDS AND RELATED SUBSTANCES

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A critical study of the amino acids was undertaken in an effort to discover the particular groups whose linkage is weakened by the absorption of light energy of different frequencies. The present paper deals with substances whose constitution varies in the number of substituent groups, CH_2, $COOH$, or SH, with consequent symmetry, or asymmetry in their spatial configuration. Our results show that total absorption of light of short wave-lengths occurs in all these substances, and selective absorption in the case of symmetrically formed molecules.

The amino acids used were HCl solutions of alanine ($CH_2 \cdot CHNH_2 \cdot COOH$), cysteine ($HS \cdot CH_2 \cdot CHNH_2 \cdot COOH$), aspartic acid ($HOOC \cdot CH_2 \cdot CHNH_2 \cdot COOH$), glutaminic acid ($HOOC \cdot CH_2 \cdot CH_2 \cdot CHNH_2 \cdot COOH$), and cystine ($HOOC \cdot CHNH_2 \cdot CH_2 \cdot S \cdot S \cdot CH_2 \cdot CHNH_2 \cdot COOH$). For purposes of comparison butyric acid ($CH_3 \cdot CH_2 \cdot CH_2 \cdot COOH$) and succinic acid ($HOOC \cdot CH_2 \cdot CH_2 \cdot COOH$) were included in the investigation. The ultra-violet absorption spectra had been previously studied to about 2100 Å. for alanine (1–3), aspartic acid (4), glutaminic acid (1), cystine (1, 3), butyric acid (5), and succinic acid (5). In the present study complete absorption spectra have been obtained from 6000–1850 Å., as stated in the preliminary reports made on our results (6, 7).

The same experimental procedure, described in a previous paper (3), was used in the visible and ultra-violet regions, and the molecular absorption coefficients were obtained.

The primary solutions from which dilutions were made contained either a 0.1 molecular weight of the substance in concentrated HCl solution, or a 0.01 molecular weight of the substance in a 1:10 dilution of HCl solution. In this way the ratio of the number of HCl molecules to the number of molecules of the dissolved substance per unit volume remained constant throughout the investigation.
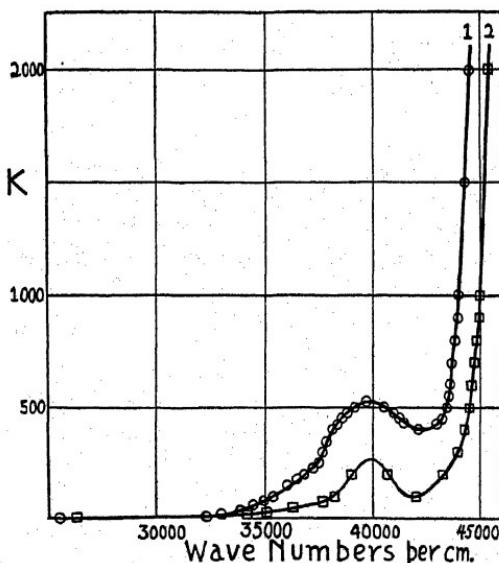
Fig. 1. Molecular absorption coefficients, K, plotted against the frequency of the absorbed light for l-cystine in HCl solution (Curve 1) and i-cystine in water (Curve 2).

In the earlier paper, the absorption curve for alanine was shown for values of the molecular absorption coefficient up to 100. By continuing the study of its absorption spectrum to shorter wavelengths, higher values for this coefficient have been obtained. A similar study of cysteine has been carried out for the first time. Comparison of the results for cystine, cysteine, and alanine revealed a lack of parallelism for cystine in the region of 2500 Å., and a more detailed investigation for l-cystine in HCl solution and i-cystine in water resulted in the discovery of a small band of selective absorption with its maximum at 2510 Å., as is seen in
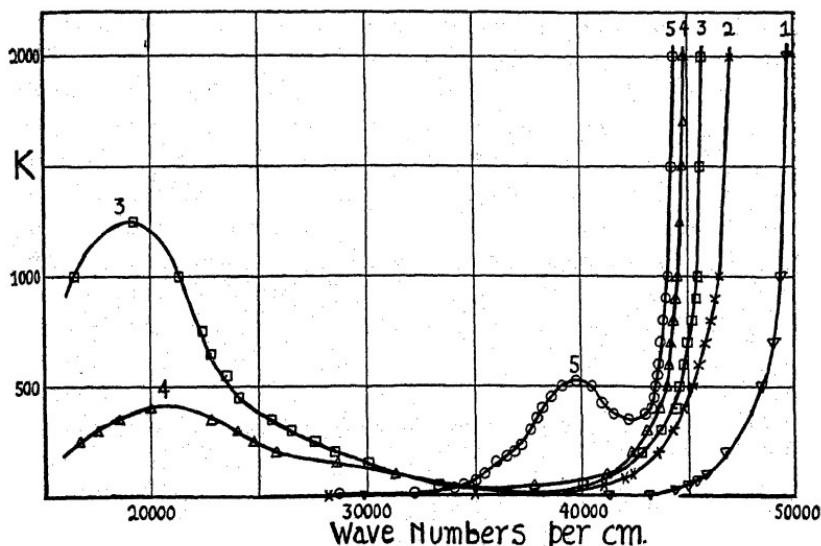
FIG. 2. Variation of molecular absorption coefficients with the frequency of the absorbed light for alanine (Curve 1), cysteine (Curve 2), aspartic acid (Curve 3), glutaminic acid (Curve 4), and *l*-cystine in HCl solutions (Curve 5).
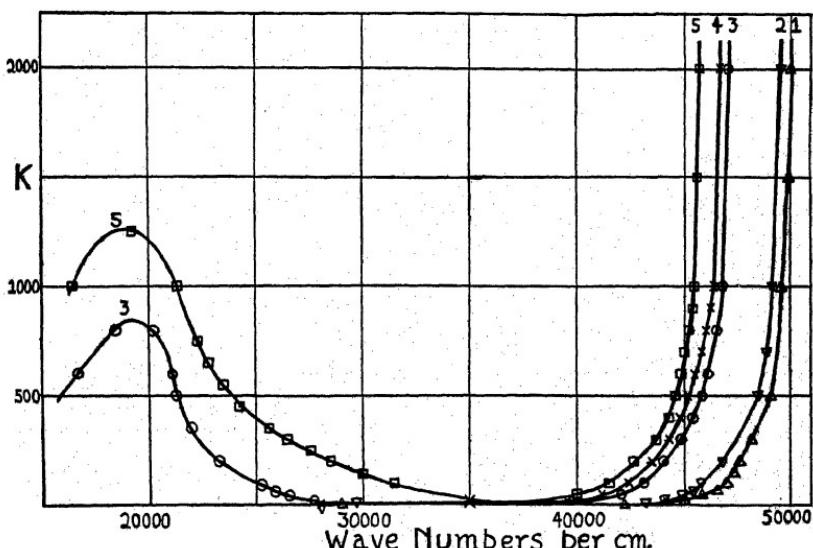
FIG. 3. Molecular absorption curves for butyric acid (Curve 1), alanine (Curve 2), succinic acid (Curve 3), cysteine (Curve 4), and aspartic acid (Curve 5).

Fig. 1. This is the same region in which Lorenz and Samuel (8) have found selective absorption in the spectra of other sulfur compounds. It was recognized that the constituent groups of cystine are arranged symmetrically about the axis between the S—S linkage and we suspected that this selective absorption might be due to the weakening of this linkage with probable disruption. This led to an extended study of the spectra of aspartic and glutaminic acids, which are nearly symmetrical about an axis between the α- and β-carbon atoms. Bands of selective absorption were found in the visible region with maxima at 5260 Å. and 4950 Å.

TABLE I

Characteristic Frequencies and Energy of Dissociation

Substance	Molecular weight	Frequency of maximum selective absorption	Frequency of edge of continuous band	Energy of dissociation
		cm.$^{-1}$	*cm.*$^{-1}$	*volts*
Alanine..........................	89		49677	6.14
Cysteine........................	121		46664	5.77
Aspartic acid....................	133	19011	45767	5.66
Glutaminic acid.................	147	20202	44883	5.55
Cystine in HCl.................	240	39841	44425	5.49
" " H$_2$O.................	240	39841	45454	5.62
Butyric acid.....................	88		49950	6.18
Succinic "	118	20408	47920	5.81

Fig. 2 shows the absorption curves obtained for these amino acids in HCl solutions.

For purposes of comparison we obtained the absorption spectrum of succinic acid whose spatial configuration is truly symmetrical about this axis. A band of selective absorption was likewise discovered for this acid in the same region with maximum at 4900 Å., as shown in Fig. 3. This confirms our interpretation of this band as being due to the absorption of energy which produces vibration in the α-β linkage.

Total absorption was obtained for all the amino acids in the ultra-violet region, between 2300 and 2000 Å., as is shown in Fig. 2. The frequency at which this appeared decreased as the molec-

ular weight of the substance increased. This absorption must be due to dissociation of some group common to all the substances under consideration, and to determine whether it occurs in the carboxyl or the amino group, a further study of butyric and succinic acids was carried out, the absorption curves obtained being parallel to those of the amino acids (see Fig. 3). This seems conclusive evidence that the dissociation occurs in the carboxyl group, as has been assumed by Ley and his collaborators (2, 9). In Table I we give the frequencies, in wave numbers, at which the maxima occur in the selective absorption produced by symmetrically formed molecules, and the frequencies at which total dissocia-
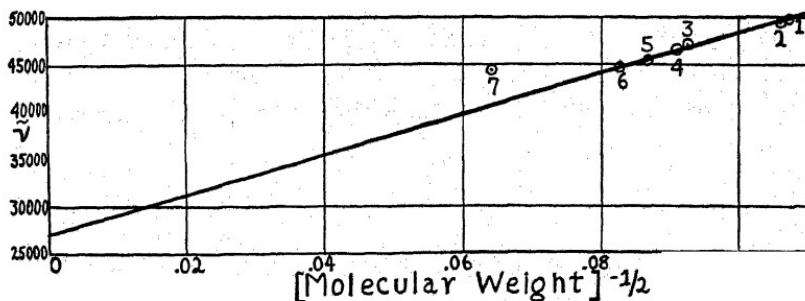
FIG. 4. The frequency of the absorbed light which dissociates the COOH group in (1) butyric acid, (2) alanine, (3) succinic acid, (4) cysteine, (5) aspartic acid, (6) glutaminic acid, and (7) cystine in HCl solutions, as a function of the reciprocal of the square root of the molecular weight of the absorbing substance.

tion is produced in all the substances studied. The dissociation energy, in volts, is calculated for each of these substances from the equation, $\dfrac{Ve}{300} = h\nu$, where V is the voltage, e the charge of the electron, h Planck's constant, and ν the frequency of the absorbed light.

The dissociation of the carboxyl group is probably due to the separation of the positive hydrogen ion from the rest of the group, and this will be accomplished when the energy absorbed is sufficient to overcome both the molecular and atomic forces which exist between the hydrogen ion and the $R \cdot CHNH_3^+ \cdot COO^-$ ion. To overcome the molecular forces, the absorbed energy must produce a large relative displacement of these ions, which results

in the vibration of the molecular ion about its position of equilibrium. When the frequency of the absorbed radiation is equal to the natural frequency of vibration of this ion the force of restitution will become insufficient to hold the ions together. The natural frequency of this vibration, v_η, is given by the equation

$$v_\eta = \frac{1}{2\pi}\sqrt{\frac{f}{m}}\tag{1}$$

where f is the elastic coefficient and m the mass of the molecular ion which is vibrating. To overcome the atomic forces the absorbed energy must be sufficient to separate the electron of hydrogen from its nucleus. Energy of frequency, v_e, must be absorbed to accomplish this result. The frequency of light which will produce dissociation will be, therefore, the sum of these two frequencies, and will be given by the equation

$$v = v_e + v_\eta\tag{2}$$

To test the validity of this equation we have plotted the wave numbers, $\bar{v} = \dfrac{v}{c}$, where c is the velocity of the light, against the reciprocal of the square root of the molecular weight of $R\cdot CHNH_3^+\cdot COO^-$ ion in Fig. 4. The graph shows that a linear relation exists between these quantities, except in the case of cystine. The slope of the line indicates that the value of the elastic constant equals 26.2×10^8, and the intercept of the line shows that $\bar{v}_e = 27,419$, which is the limiting wave number of the Balmer series in the spectrum of hydrogen. The frequency at which total dissociation occurs is, therefore, given by the equation

$$\bar{v} = 27,419 + \frac{1}{2\pi} \times \frac{5.12 \times 10^4}{\sqrt{m}}\tag{3}$$

The previous interpretation of the shift of absorption spectra of similar molecules toward the red with increasing molecular weight was that of Henri (10), who assumed that the change in the frequency of the light which produced total dissociation was proportional to the number of groups in the molecule. This assumption does not lead to the linear relationship which we have shown exists between the frequency of the absorbed light and the reciprocal of the square root of the mass of the molecule.

The fact that the frequency of the light energy which produced the dissociation of the carboxyl group in cystine did not appear to obey this law led us to further study of our curves. The molecular weight of cystine indicates that the frequency at which this would be expected to occur, when calculated from Equation 3, is approximately the same as that which results in selective absorption. Light of this frequency weakens the S—S linkage, and if this causes the molecule to dissociate into ions, *i.e* into R—S$^+$ and R—S$^-$, in the presence of hydrochloric acid, cysteine chloride and cysteine will be formed. The molecule of higher molecular weight will then be dissociated in the carboxyl group by energy of the smaller frequency, which will be revealed in the absorption curve. With the molecular weight of this molecule, 155, cysteine chloride is found to obey Equation 3.

To test the presence of cysteine chloride and cysteine in irradiated solutions, the mean molecular weight of the solute was determined by the lowering of the freezing point of water, with the Raoult equation, where the mean molecular weight, $\overline{M}$, is given by the equation

$$\overline{M} = \frac{RT_0^2}{L\rho V} \times \frac{x}{\Delta T} = k\frac{x}{\Delta T} \tag{4}$$

where k is known as the Raoult coefficient and equals 1.858 per 100 cc. for water.

If we take as our unit of volume that which contains, on the average, 1 cystine molecule, there will be y HCl molecules in the same volume, since we maintained a constant ratio, y, between the concentrations of HCl and cystine in the solution. Hence, the mean molecular weight of the molecules in the solute will be given by the equation

$$\overline{M} = \frac{\Sigma M}{N} \tag{5}$$

where ΣM equals the sum of the molecular weights of the cystine and the y HCl molecules in the unit and N is the number of particles. Since $x = C\Sigma M$, where C is the concentration of the cystine, by combining Equations 4 and 5 we obtain

$$N = \frac{\Delta T}{1.858\ C} \tag{6}$$

By determining the lowering of the freezing point, 0.425°, produced by the hydrochloric acid solution of concentration 0.1165 M, it was found that in this dilute solution the hydrochloric acid was almost totally dissociated. Therefore, in each unit there would be $2y$ ions to each cystine molecule, making $2y + 1$ particles in the unit. If there is combination between these particles, assum-

TABLE II

Number of Particles per Cystine Molecule in HCl Solutions

Experiment	Concentration of cystine	$2y$	ΔT	No. of particles
	M		°C.	
A. Before irradiation	0.01	23.3	0.417	22.5
	0.001	233.0	0.430	231.6
B. After irradiation through filter	0.01	23.3	0.407	21.9
	0.001	233.0	0.428	230.3
C. After irradiation without filter	0.01	23.3	0.430	23.2
	0.001	233.0	0.432	232.5

ing Bjerrum's (11) theory that amino acids exist in the *Zwitter Ion* form, such combination may occur in one of the following ways.

yH$^+$ + yCl$^-$ + $^-$OOC·NH$_3^+$HC·CH$_2$·S·S·CH$_2$·CHNH$_3^+$·COO$^-$→

(a) $(y - 1)$H$^+$ + yCl$^-$ + HOOC·NH$_3^+$HC·CH$_2$·S·S·CH$_2$·CHNH$_3^+$·COO$^-$ or

(b) $(y - 2)$H$^+$ + yCl$^-$ + HOOC·NH$_3^+$HC·CH$_2$·S·S·CH$_2$·CHNH$_3^+$·COOH

or

(c) $(y - 2)$H$^+$ + $(y - 2)$Cl$^-$ + HOOC·NH$_3^+$ClHC·CH$_2$·S·S·CH$_2$·CHNH$_3^+$-Cl·COOH
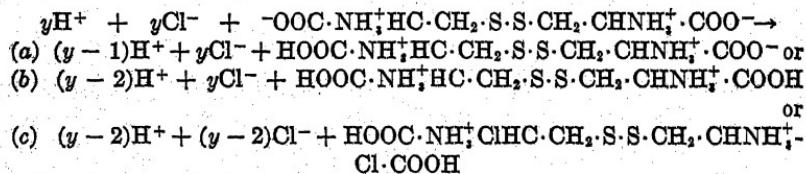
Combination (a) would result in $2y$ particles in the unit; (b) would result in $2y - 1$ particles in the unit; (c) would result in $2y - 3$ particles in the same unit.

The most probable combination was determined for two solutions, 0.01 M and 0.001 M cystine, each in 0.1165 M HCl solution, and the results obtained in both cases indicated, within experimental error, the existence of $2y - 1$ particles in the unit, as is shown in Experiment A of Table II. This indicates that cystine in HCl solutions forms a doubly charged positive ion.

If upon irradiation the S—S bond is broken with the consequent

formation of cysteine chloride and cysteine, there will be $2y - 2$ particles in the unit. After irradiation for 2 minutes by light which had passed through a Wratten filter No. 17, which isolates light of the frequency range necessary to produce this dissociation, the number of particles in the unit was again calculated and proved to be $2y - 2$, as is shown in Experiment B of Table II.

When irradiated by unfiltered light from the hydrogen discharge tube the carboxyl group in each of these molecules will also be dissociated, freeing 2 positive hydrogen ions, and consequently there will be $2y$ particles in the unit. This hypothesis was also tested and its validity is shown in the results tabulated in Experiment C of Table II.

All of these results agree with the theory that cystine in HCl solution is dissociated at the —S—S— linkage by the absorption of light of wave-lengths nearly equal to 2500 Å., with the consequent formation of cysteine chloride and cysteine. Assuming a similar dissociation to occur when i-cystine is dissolved in water and irradiated with light of this wave-length, cysteine hydroxide and cysteine will be formed when the —S—S— linkage is broken. When the wave number of the light which produces dissociation of the carboxyl group is substituted in Equation 3, a molecular weight of about 137 is predicted for the molecule which is dissociated. This is the molecular weight of cysteine hydroxide.

SUMMARY

In this study of the absorption spectra of selected amino acids and related compounds we have found:

1. Symmetrically formed molecules absorbed light energy selectively, weakening the linkage between the α,β-carbon groups, as in the case of aspartic, glutaminic, and succinic acids, and between the —S—S— groups in cystine.

2. In the case of cystine in HCl solution this disruption results in the formation of cysteine chloride and cysteine, and in water, cysteine hydroxide and cysteine.

3. The frequency of the light which produces dissociation of the carboxyl group is the sum of the electronic frequency, ν_e, in this case the limiting frequency of the Balmer series of hydrogen, and of the natural frequency of vibration, ν_η, of the molecular ion.

Our thanks are due to Miss Charlotte Klingler, who measured the freezing points of the various cystine solutions discussed in this paper.

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DETERMINATION OF SMALL AMOUNTS OF ETHYL IODIDE

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The use of ethyl iodide in the indirect determination of cardiac output in man gives importance to methods for the determination of low concentrations of its vapor in air. The iodine pentoxide method advocated by Henderson and Haggard (1) was found by Starr and Gamble (2) to give variable results, depending on the sample of iodine pentoxide used and the rate at which the ethyl iodide-air mixture was passed through it. The silver nitrate method developed by Starr and Gamble (2) gave results of a relatively high degree of accuracy; average error, 0.03 mg. of ethyl iodide in a 500 cc. air sample. This probable analytical error, however, introduces an error of 6 to 7 per cent in the calculated cardiac output ((3) p. 471). Further efforts in the direction of greater analytical accuracy seemed advisable.

In the method described in this paper the ethyl iodide is treated with chlorine, or bromine, which promptly oxidizes the iodide to iodate (4, 5). The excess halogen is removed with phenol and the iodine, liberated from potassium iodide by the iodate, is determined by titration with thiosulfate. With vaporized samples of the order of 3 mg. the average error was only 0.5 per cent (Table II) in terms of ethyl iodide, equivalent to an error of only 2 to 3 per cent in the value for cardiac·output as determined by the method of Starr and Gamble (3). The advantages of the method are the definiteness of the titration end-point, the sixfold increase in amount of iodine titrated, and the elimination of the 16 to 24 hours delay necessary in the Starr and Gamble method (2) for the completion of the ethyl iodide-silver nitrate reaction.

Procedure

The procedure applicable to air samples containing vaporized ethyl iodide is as follows: The ethyl iodide-air mixtures are collected in 500 cc. gas sampling tubes previously described (1–3). 10 cc. of chlorine water[1] not more than a day old are placed in a reagent tube[2] which is inserted into the end of one of the rubber connections on the sampling tube so that the pinch-clamp can be removed without allowing gas to escape. After removing the pinch-clamp, the mouth of the reagent tube is pushed through the rubber tubing until it is 2 to 3 cm. within the glass neck of the sampling tube. The chlorine water is allowed to run into the main body of the sampling tube, but not into the opposite neck. The tube is leveled and rotated so that the walls are washed with the reagent. The contents of the reagent and sampling tubes are washed into an Erlenmeyer flask with a total of 30 to 35 cc. of distilled water divided into at least three portions. The liquid in the flask is set into rotation and 10 cc. of 10 per cent phenol-water mixture are added rapidly from a graduated cylinder.[3] After standing for at least 20 minutes, 25 cc. of water and 5 cc. of 20 per cent potassium iodide solution, which has been prepared on the day it is to be used, are added, and the iodine liberated is titrated, at once, with 0.005 N sodium thiosulfate.[4] When the

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[1] The chlorine water used in testing the method contained approximately 0.35 per cent of Cl. Since the concentration need be known only approximately, the strength of the reagent can be judged with sufficient accuracy by its appearance. *Cf.* The Pharmacopœia of the United States of America, tenth decennial revision, Philadelphia, 487 (1926).

[2] The reagent tubes were made from glass tubing the outside diameter of which was slightly less than the inside diameter of the neck of the sampling tube. At one end a 10 cc. bulb was blown, giving the tube the appearance of a small retort.

[3] If the phenol is added slowly it is partially oxidized, producing colored compounds (Kendall ((6) p. 896)). In such cases high values for ethyl iodide were obtained.

[4] When the highest analytical accuracy is required, as in the tests which follow, the 0.005 N sodium thiosulfate must be made with recently boiled distilled water, must not be more than 2 weeks old, and must be standardized each day. If proportionate concentrations only are required, as in the application to the cardiac output method, standardization is unnecessary. The stability of 0.1 N thiosulfate solution is much greater than that of the weaker solution and may conveniently be used as a stock solution.

yellow color of the iodine has nearly disappeared, 5 cc. of 0.2 per cent starch solution are added and the titration is continued until the solution is colorless. 1 cc. of 0.005 N sodium thiosulfate is equivalent to 0.130 mg. of ethyl iodide in the original sample.

5 cc. of approximately saturated bromine water[5] may be used, instead of the 10 cc. of chlorine water, in the above procedure and the excess removed with 5 cc. of 10 per cent phenol.

Tests of Method

The accuracy of the method has been proved by the following experiments.

Eastman's ethyl iodide was redistilled, first over powdered silver, then over mercury. Samples weighing from 1 to 12 mg. were introduced into weighed capillary tubes or bulbs with capillary stems. These were sealed after waiting until the ethyl iodide at the end of the capillary had evaporated, and weighed again on the following day. The weighings were made on an assay balance with weights calibrated by Richards' method (7); the average change in weight from handling and sealing alone (determined by eight experiments) was ±0.007 mg. The capillary tube, or bulb, was placed in an Erlenmeyer flask containing 25 cc. of water plus 10 cc. of freshly prepared chlorine water and thoroughly crushed with a glass rod. The flask was rotated while 10 cc. of 10 per cent phenol in water were added rapidly. After standing for at least 20 minutes, 25 cc. of water and 5 cc. of freshly prepared 20 per cent potassium iodide solution were added and the liberated iodine was titrated with 0.005 N sodium thiosulfate, 5 cc. of 0.2 per cent starch solution being used as indicator.

The sodium thiosulfate solutions, never more than 2 weeks old, were standardized each day that they were used. Bureau of Standards arsenious oxide was used as the ultimate standard, while standard potassium biiodate, which was compared with the arsenious oxide several times during the investigation, was used for daily standardizations. Blanks were run on all the reagents and the distilled water used. Standardizations, comparisons, and blanks were made under the same conditions as the

[5] The bromine water used contained approximately 2.5 per cent of Br (cf. foot-note 1).

determinations. Solution volumes were corrected for temperature.

The results of consecutive determinations, made on 2 different days, are given in Table I. The greatest error in this series is +1.8 per cent, the arithmetical mean of the errors is +0.4 per cent, and the average deviation from this ±0.6 per cent. In other series of analyses of ethyl iodide, in accurately measured volumes of water solutions, evidence was obtained that the errors involved

TABLE I

Determination of Weighed Samples of Ethyl Iodide Released under Chlorine Water in Erlenmeyer Flask

Excess chlorine was removed with phenol.

Sample taken	Found	Error
mg.	*mg.*	*per cent*
4.390*	4.380	−0.2
6.048	6.083	+0.6
12.516	12.354	−1.3
3.425	3.414	−0.3
4.370	4.379	+0.2
3.894*	3.930	+0.9
2.245*	2.285	+1.8
1.704	1.721	+1.0
1.744	1.752	+0.5
1.055	1.057	+0.2
2.063	2.080	+0.8
3.456*	3.460	+0.1
Arithmetical mean...........................		+0.4
Average deviation from arithmetical mean....		±0.6

* 20 cc. of chlorine water and 20 cc. of 10 per cent phenol were used.

in weighing the ethyl iodide samples were an important factor in the inconsistencies, small as they were, in these results. A solution of ethyl iodide was prepared containing approximately 0.58 mg. in 10 cc. Analyses in triplicate (two with chlorine, one with bromine) were made of 10, 20, 30, 40, and 50 cc. samples, the same 10 cc. pipette being used in each measurement. Each result was compared with the amount of ethyl iodide calculated to be present from the average of all fifteen determinations. The maximum deviation from the arithmetical mean of the series was +0.004

mg., and the average deviation from the arithmetical mean was only ±0.002 mg.

In order to test the method under conditions which more closely resembled those present in measurements of cardiac output, vaporized samples of ethyl iodide were used. Samples weighed in capillary tubes, as described above, were placed in 500 cc. gas sampling tubes, similar to those used by Henderson and Haggard ((1) Fig. 6) and Starr and Gamble (2, 3), together with a piece of glass rod. The ends of the tube were closed by rubber tubing and

TABLE II

Determination of Weighed Samples of Ethyl Iodide Vaporized in 500 Cc. Gas Sampling Tubes

The samples were oxidized with chlorine water, and excess chlorine was removed with phenol.

Sample taken	Found	Error
mg.	*mg.*	*per cent*
2.346	2.375	+1.2
3.221	3.278	+1.8
3.590	3.613	+0.6
4.463	4.496	+0.7
1.651	1.686	+2.1
2.168	2.177	+0.4
2.377	2.398	+0.9
2.822	2.827	+0.2
3.731	3.754	+0.6
Arithmetical mean................................		+0.9
Average deviation from arithmetical mean.....		±0.5

Mohr pinch-clamps, and the capillary was thoroughly crushed by whirling the glass rod around inside the sampling tube. When the ethyl iodide had vaporized, the analysis was carried out as described before under "Procedure."

The results of nine consecutive experiments, performed on 2 different days are given in Table II. With seven weighed samples of the same order of magnitude analyzed with bromine water as the oxidizing agent the greatest error was +1.5 per cent; the arithmetical mean of the errors was +0.001 mg. or +0.2 per cent, and the average deviation from the arithmetical mean ±0.011 mg. or 0.5 per cent.

The method described above is at once directly applicable as an improvement in the analytical technique of the Starr and Gamble cardiac output method. In future developments of the mechanical features of that method it may be found feasible to pass the air to be analyzed directly through chlorine water. Preliminary tests have shown that the absorption of ethyl iodide is rapid and probably adequate to meet the requirements of such a modification of the method.

The author is indebted to Dr. C. J. Gamble for valuable advice throughout the course of this work.

SUMMARY

Low concentrations of ethyl iodide in air and water have been determined by an iodate method in which chlorine or bromine is used as an oxidizing agent, the excess being removed with phenol.

In the range of quantities of ethyl iodide dealt with in an indirect determination of cardiac output the average error was 0.011 mg. This is approximately one-third the error found by Starr and Gamble in their silver nitrate method, and may be held responsible for an error of only 2 to 3 per cent in the value for cardiac output.

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PRODUCTION OF VITAMIN A BY A SPECIES OF CORYNEBACTERIUM*

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Although vitamin B (presumably both vitamins B and G in the American terminology) is known to be produced by a number of bacteria (8), there is no convincing record encountered by the authors of synthesis of vitamin A by bacteria. Wollman and Vagliano (10) found no evidence of vitamin A in *Lactobacillus bulgaricus*. Slanetz (7) also reports (without data) no evidence of vitamin A production by *Azotobacter chroococcum, Bacterium lactis acidi, Bacterium radicicola, Bacterium prodigeosum, Bacillus subtilis, Bacillus mycoides, Micrococcus agilis,* or three unidentified soil bacteria. Cunningham (1) was unable to demonstrate synthesis of vitamin A by three strains of *Mycobacterium tuberculosis,* but due to the prolonged heating to which the organisms were subjected it is possible that vitamin A, if present, would have been at least partially destroyed.

It was thought, when the work was started, that some idea of the structure of vitamins could be obtained by growing bacteria on vitamin-free substrate, and that, by adding various chemicals or altering the conditions of growth (3), the factors necessary for the production of the vitamins might be determined. Lately it has been rather generally accepted that carotene may be transformed in the animal body into vitamin A (2, 5), although it is well established that the two substances are not identical (6).

The methods accepted were those described by Gunderson and Skinner (3). The medium consisted of dextrose (Difco) 5 gm., agar (Difco) 15 gm., peptone (Parke, Davis and Company) 5 gm., and distilled water 1000 cc.

The agar was ground and washed continuously with alcohol for 72 hours, followed by ether for the same length of time The peptone was washed with ether only. Cultures were incubated at room temperature in total darkness. The preparation of the growth was as described in the paper mentioned above. The organism used was an unidentified diphtheroid, *Corynebacterium* sp., cultures of which are maintained in this department.
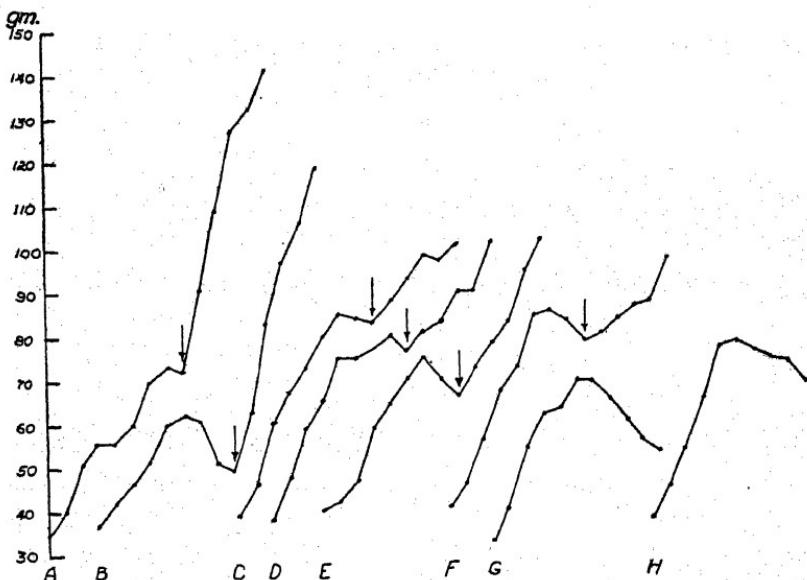
Fɪɢ. 1. Weight, at weekly intervals, of rats on vitamin A-poor diet. Rats A and B were given 1 drop of cod liver oil per day, after the point indicated. Rats C, D, E, and F were given 1 gm. of dried bacteria per day, after the point indicated. Rats G and H were given no curative. Both died before conclusion of experiment, with severe xerophthalmia.

The rats used were from a colony which had been maintained by Dr. Mildred Ziegler of the Department of Pediatrics for several generations on a diet considered border-line as to vitamins A and D, and, although healthy, they were small. They were originally from the Jackson colony maintained in the Institute of Anatomy. At the age of 28 days the rats were put on a diet designed to produce vitamin A deficiency symptoms (9). To the diet described was added 1 per cent of agar for roughage. Crisco, a vegetable vitamin A-free fat, was substituted for lard. When the typical

vitamin A deficiency symptoms appeared, as evinced by loss of weight and by xerophthalmia, each animal was given one of three treatments: 1 drop of cod liver oil per day, no supplement, or 1 gm. of dried bacterial growth. A preliminary experiment had demonstrated that the rats would eat 1 gm. amounts of bacteria readily, but not always $1\frac{1}{2}$ gm. Weekly weights of the rats were taken, the results of which are seen in Fig. 1, which represents two separate experiments. Records of the food consumed were made weekly.

To insure that the effects noted did not result from traces of the vitamin in the medium, control experiments were run. After the animals showed xerophthalmia symptoms and loss of weight, these were divided into four lots: Lot 1, receiving no further treatment; Lot 2, receiving cod liver oil; Lot 3, receiving a diet in which half the casein (Harris) was replaced by the peptone, and half the dextrin by dextrose; Lot 4, as Lot 3, but also with cod liver oil. The results[1] indicate that no significant amount of vitamin A was present in the ingredients of the culture medium.

Fig. 1 shows that this organism produced vitamin A, but not in large amounts. There was a slow but definite gain in weight with a prompt cure of the xerophthalmia, whereas control animals continued to lose weight and died. It is also evident that the vitamin was synthesized and did not originate in impurities in the medium.

It is not known whether the organism used produced carotene. It has an orange color, but little is known as to the carotene production by bacteria (6). If it can be shown that the organism used contains carotene, these experiments offer added evidence

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[1] The following figures represent the weekly weights of the rats in gm. The asterisk indicates the point at which the modification of the diet was commenced.

Lot 1. Rat 1.   40, 59, 71, 83, 87, 93, 91, 72, died
           2.   38, 56, 67, 86, 92, 90, 85, 65,   "
Lot 2.   1.   43, 62, 81, 100, 105, 112, 99,* 125, 155, 177, 183, 193
           2.   41, 59, 69, 83, 91, 89,* 104, 121, 131, 144, 159, 170
Lot 3.   1.   41, 64, 76, 88, 94, 94, 92,* 67, died
           2.   47, 65, 79, 94, 112, 105, 92,* 60, "
Lot 4.   1.   39, 55, 71, 85, 84, 82,* 90, 121, 138, 157, 175, 192
           2.   43, 55, 76, 85, 92, 70, 69,* 81, 121, 145, 175, 185
           3.   41, 55, 66, 81, 90, 83,* 108, 132, 145, 156, 168, 176

that carotene is the provitamin A. If not, it is possible that the vitamin only recently said to be purified (4) may be obtained from microbial sources unaccompanied by carotene, an accomplishment not as yet attained in any member of the plant kingdom. Cultures of the organism will be supplied to any one who desires them.

SUMMARY

1. Dried bacterial growth of a species of *Corynebacterium* grown on a vitamin A-free substrate in total darkness cured young rats suffering from vitamin A deficiency. Xerophthalmia was cured promptly, and a slight but definite increase in weight resulted when the bacteria were fed in 1 gm. amounts daily as a supplement to the diet which produced the symptoms.

2. The control animals all lost weight rapidly and died.

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STROPHANTHIN

XXVI. A FURTHER STUDY OF THE DEHYDROGENATION OF STROPHANTHIDIN

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The application of the selenium method of Diels, Gädke, and Körding[1] to the dehydrogenation of strophanthidin yielded a mixture of aromatic hydrocarbons which was described in an earlier note.[2] More recent attempts have been made to separate the individual products of the reaction for further investigation and possible identification with known aromatic hydrocarbons. Although larger amounts of strophanthidin were employed in our experiments, it has not been found possible to secure a certain or satisfactory separation of the products of the reaction. The various hydrocarbon fractions obtained on distillation were submitted to repeated fractionation and recrystallization, as well as conversion into picrates. The resulting substances were apparently still mixtures with which it was unprofitable to proceed further, with the amounts available. However, in the case of one of these substances a more extensive study became necessary. This substance was the one previously described as melting after repeated recrystallization at 130–134°. From the analytical results a formula $C_{18}H_{16}$ was then suggested. In our more recent work, however, by careful fractionation the substance has been obtained in a form which gave the appearance of greater homogeneity and which melted more sharply at 125–126°. Since Diels, Gädke, and Körding had already described a substance of similar character and melting point, which was obtained by dehydrogenation of cholesteryl chloride, it was of interest to make a comparison

[1] Diels, O., Gädke, W., and Körding, P., *Ann. Chem.*, **459**, 14 (1927).
[2] Jacobs, W. A., and Fleck, E. E., *Science*, **73**, 133 (1931).

of the substances obtained from both sources. For this purpose we have repeated the preparation of the cholesterol hydrocarbon according to the directions of these workers. At first the impression was gained that the strophanthidin and cholesterol derivatives were identical, since their crystalline form appeared to be indistinguishable and no depression in melting point could be obtained on mixing the two. However, further investigation showed that they were not identical. In the first place, instead of the blue fluorescence in ultra-violet light shown by the crystals of the cholesterol compound, the strophanthidin derivative gave a violet fluorescence, a property which was gradually lost on standing. Further, the picrates of each substance showed different melting points. Finally, on oxidation with chromic acid the two substances behaved quite differently. In the case of the cholesterol hydrocarbon, we have confirmed the observation of Diels et al. that no characteristic reaction product is produced by this reagent. The strophanthidin derivative, however, gave a red quinone. The analytical figures obtained with the latter, as well as with the hydrocarbon itself, have made necessary a revision of the formula of the latter to $C_{16}H_{14}$. The red quinone, $C_{16}H_{12}O_2$, gave a quinoxaline derivative with o-phenylenediamine with the formula $C_{22}H_{16}N_2$. The hydrocarbon is possibly a dimethylphenanthrene. Of the numerous possible dimethylphenanthrenes only a few have been described. Our substance does not appear to conform to the description of any of these. It is possible that the determination of the identity of the strophanthidin hydrocarbon will be of significance in interpreting the structure of the parent substance.

We have not succeeded in obtaining chrysene from the mixture of higher melting hydrocarbons. The melting point of the fraction, which approached that of chrysene, was definitely depressed when the two were mixed.

The products of the dehydrogenation of strophanthidin appear, therefore, to be definitely different from those obtained from cholesterol. To use this, however, as an argument that the molecular skeletons are entirely different is precarious, especially in view of the experience of Diels and Karstens[3] who have found that the dehydrogenation of cholic acid with selenium takes a different course than in the case of cholesteryl chloride.[1]

[3] Diels, O., and Karstens, A., *Ann. Chem.*, **478**, 129 (1930).

A mixture of 50 gm. of strophanthidin and 70 gm. of selenium was heated at 340–350° for 30 hours. The chloroform extract of the reaction mass gave a resinous residue after removal of the solvent. On distillation at 2 mm. pressure a partly crystalline oil was obtained. 47 gm. of this material were obtained from a total of 250 gm. of strophanthidin. This mixture was then submitted to repeated fractionation at 2 mm. and finally gave the following: Fraction I, 160° or below, 3.5 gm.; Fraction II, 160–175°, 2.7 gm.; Fraction III, 175–190°, 1.7 gm.; Fraction IV, 190–200°, 9.0 gm.; Fraction V, 200–210°, 4.1 gm.; Fraction VI, 210–220°, 7.1 gm.; Fraction VII, 220–230°, 4.8 gm.; Fraction VIII, 230–245°, 3.0 gm.; Fraction IX, 245–260°, 2.8 gm.; Fraction X, 260° or above, 2.9 gm.

The lower boiling fractions consisted of a mixture of crystals and oil, while in the higher boiling fractions the crystals were imbedded in a thicker, more resinous material. Each fraction was, therefore, converted into a picrate. Fractions I to VI yielded picrates from alcoholic picric acid solution, and Fractions VII to X gave picrates from a benzene solution of picric acid. In each case the picrates were repeatedly recrystallized from such picric acid solutions. The picrates were then decomposed with dilute ammonia and the hydrocarbons were extracted with chloroform. The residues obtained after evaporation of the solvent were recrystallized from 95 per cent alcohol. The yields and melting points of the hydrocarbons obtained in this manner from each of the fractions were as follows: Fraction I, 65–75°, 0.7 gm.; Fraction II, 105–110°, 0.5 gm.; Fraction III, 120–125°, 0.27 gm.; Fraction IV, 120–130°, 1.05 gm.; Fraction V, 130–140°, 0.35 gm.; Fraction VI, 153–162°, 0.69 gm.; Fraction VII, 225–230°, 0.1 gm.; Fraction VIII, 230–235°, 0.14 gm.; Fraction IX, 242–250°, 0.17 gm.; Fraction X, 295–302°, 0.1 gm.

Fraction IV was carefully fractionated by recrystallization from 95 per cent alcohol. The least soluble, higher melting fractions were first removed. Attention was concentrated on the intermediate fraction, the melting point of which was sharpened by the process and gave the definite impression that a more homogeneous material was being secured. After repeated recrystallization from alcohol, 0.25 gm. of hydrocarbon was obtained which melted at 125–126°.

4.313 mg. substance: 2.620 mg. H_2O, 14.725 mg. CO_2
5.083 "   "   : 3.180 "   "   17.385 "   "
$C_{16}H_{14}$.   Calculated.   C 93.15, H 6.85
Found.   " 93.10, " 6.80
" 93.28, " 7.00

When freshly obtained this hydrocarbon fluoresced with a bright blue-violet color in ultra-violet light.   After 2 months, when the crystals had become discolored somewhat by a light brown, the substance no longer showed this fluorescence in the ultra-violet. Recrystallization restored this property somewhat.   However, it never reached the intensity of the original.   We convinced ourselves that the fluorescence was not due to a substance which remained in the mother liquors.

When this hydrocarbon was mixed with the hydrocarbon, $C_{18}H_{16}$, prepared according to Diels, Gädke, and Körding[1] from cholesteryl chloride and which melted at 123–125°, no depression could be detected.   In ultra-violet light the cholesterol hydrocarbon gave the characteristic blue fluorescence described by its discoverers.

The strophanthidin hydrocarbon gave a picrate with alcoholic picric acid which formed light orange needles and melted at 138–140°.   As given by Diels, Gädke, and Körding and confirmed by us, the cholesterol hydrocarbon gave a picrate which melted at 118–120°.

40 mg. of the strophanthidin hydrocarbon which melted at 125–126° were dissolved in 0.5 cc. of acetic acid and were treated with 90 mg. of chromic acid dissolved in 0.12 cc. of water and 0.6 cc. of acetic acid.   The mixture was heated at 100° for 3 hours.   After dilution dark red prisms slowly deposited in the mixture.   The yield was 15 mg. of substance which melted at 195–200° as directly obtained.   After several recrystallizations from acetic acid this was raised to 205–208°.   Owing to insufficient material this operation could not be carried to the logical point of constant melting point.

3.304 mg. substance: 1.550 mg. H_2O, 9.810 mg. CO_2
$C_{16}H_{12}O_2$.   Calculated.   C 81.32, H 5.12
Found.   " 80.98, " 5.25

It was found possible to obtain more of the quinone by the direct oxidation of the crystalline hydrocarbon Fractions I, II, and III.

Fraction I gave a quinone indistinguishable from the above, obtained from Fraction IV, and after repeated recrystallization from acetic acid melted at 208–210°.

4.213 mg. substance: 1.930 mg. H_2O, 12.580 mg. CO_2
4.227 "   "   : 1.880 "   "   12.585 "   "
Found.   C 81.44, H 5.13
" 81.20, " 4.98

Fractions II and III gave more of the same substance. The fact that the hydrocarbon which yields this quinone was found in these different hydrocarbon fractions shows the inadequacy of the repeated fractional distillations employed for the preliminary separation of these fractions.

The quinone was further characterized by conversion into the quinoxaline derivative. An acetic acid solution of the quinone was refluxed for 1 hour with the calculated amount of o-phenylenediamine. The yellow needles which separated from the cooled solution were recrystallized from chloroform-alcohol and melted at 187–188°.

4.182 mg. substance: 1.870 mg. H_2O, 13.100 mg. CO_2
4.527 "   "   : 1.960 "   "   14.200 "   "
$C_{22}H_{16}N_2$.   Calculated.   C 85.69, H 5.23
Found.   " 85.43, " 5.00
" 85.55, " 4.84
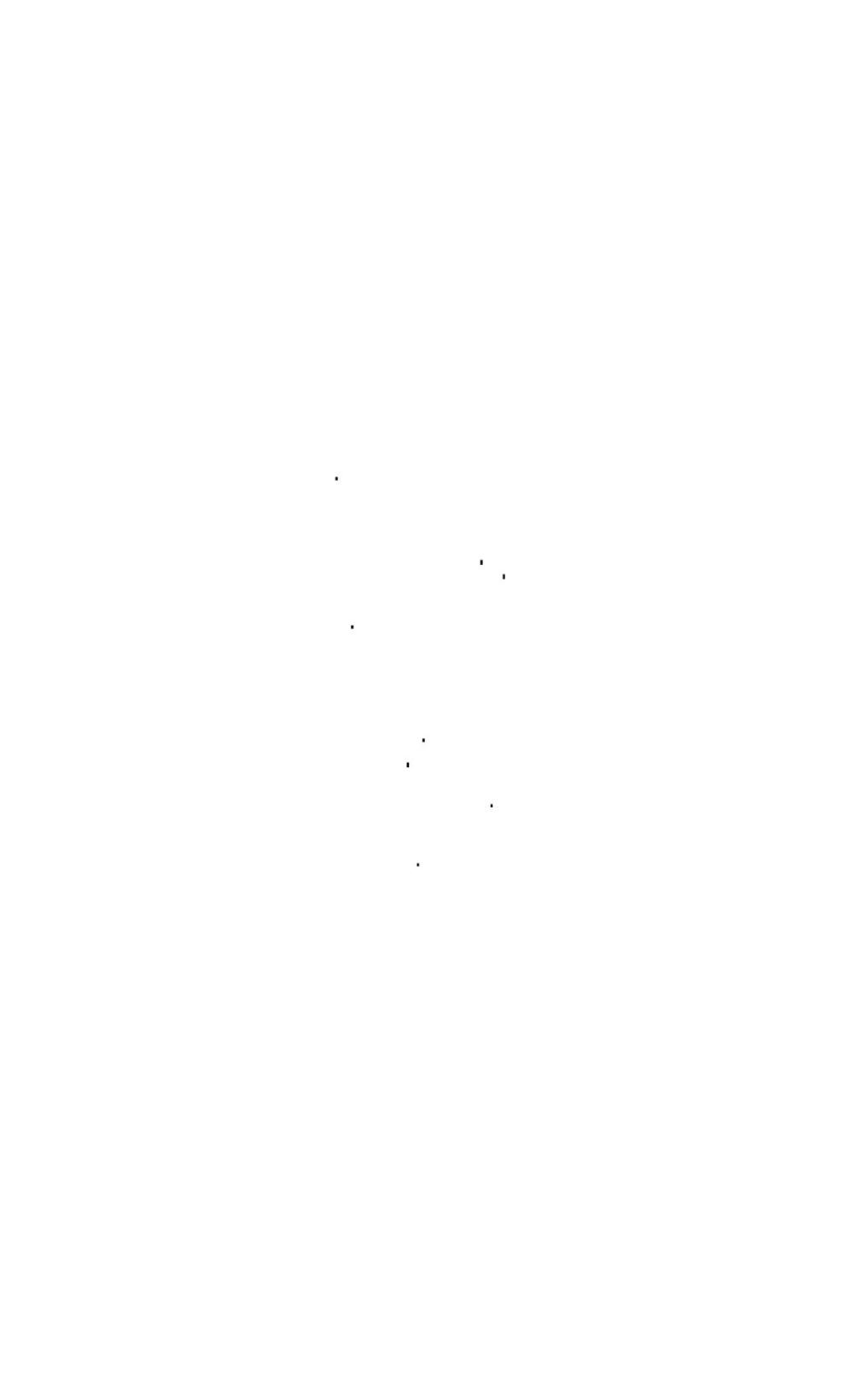
IRRADIATED MILK: THE AMOUNT OF ENERGY REQUIRED TO PREVENT RICKETS IN CHICKENS

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AND M. J. DORCAS

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The introduction and increasing utilization of antirachitic foods and pharmaceuticals have necessitated the standardization of a suitable technique for the vitamin D assay of such products. Cod liver oil has been widely accepted as the standard of comparison, and the rat unit has been generally designated as the unit of evaluation. The technique and the standard unit are entirely empirical, and as yet sufficient data are not available to permit direct interpretation of the results from different products in terms of practical therapeutic value. Certain data already available indicate that the standard unit has a variable value in practical use for the prevention of rickets in infants and chickens. From the work of Hess (1), of Massengale (2), and of Supplee (3), and their associates, the potency of the rat unit in the prevention of rickets in infants and chickens appears to be greater in activated milk than in viosterol or cod liver oil, the unit in viosterol being considerably less effective than in the other two products. It appears, therefore, that the empirical method of assay has limitations as a practical measure of the antirachitic effectiveness of different substances.

Since the quantitative determination of vitamin D by direct analytical methods is impractical, if not impossible, at the present time, a correlation of the results from the standard method with those obtained from direct use would seem to be an ultimate necessity for accurately appraising the antirachitic and calcifying properties of different dietary and therapeutic substances. The

data already published by Supplee, Dorcas, and Hess and their coworkers (3–5), wherein the radiant energy applied to milk under standardized conditions has been correlated with the empirically determined antirachitic properties, seemed to provide a basis for obtaining direct evidence of the practical prophylactic value of such milk.

<div align="center">EXPERIMENTAL</div>

Since the purpose of these tests was to determine the rickets-preventing properties of different samples of dry milk activated with a known kind and amount of energy (4, 5), the preventive technique with chickens previously described (3) was used. The test rations were composed of 22.5 parts of the activated milk (12 per cent butter fat); flour wheat middlings, 20 parts; yellow corn-meal (whole corn), 55 parts; steamed bone-meal, 2 parts; and sodium chloride, 0.5 part. Day old white Leghorn chicks, hatched from a standardized flock maintained on a standardized ration, were obtained from Cornell University. Groups of twenty-five chicks were used for each test. At the end of the 8 weeks test period the diagnostic methods and examinations previously employed were applied. Food consumption records were carefully maintained and the average amount of the test milks ingested per chick per day was calculated. For convenience of comparison with data previously obtained, the amount of milk consumed has been calculated to the original fluid milk basis.

From the records shown in Table I it will be noted that those milks which received the lower applications of energy did not possess sufficient antirachitic potency to prevent rickets in chickens under the conditions of these tests. The data show that up to a certain limit increasing amounts of energy brought about an improvement in the antirachitic value of the milk. After a given amount of energy had been applied complete protection from rickets resulted; further increases in the amount of energy were not consistently reflected in superior growth of the chickens, increased bone ash, or abnormal calcium and phosphorus content of the blood.

Those milks which had received 1328×10^8 ergs or more per cc. contained sufficient vitamin D to afford complete protection. According to the data previously reported (5) milk to which 1328

TABLE I

Relationship between Radiant Energy (2000 to 3000 Å.) Applied to Milk and Its Antirachitic Properties As Determined by Prevention of Rickets in Chickens

Group No.	Sample No.	Arc	Exposure period	Energy per cc. milk		Average milk fed per chick per day	Average energy per day per 100 gm. body weight		Final average weight	Rickets (post-mortem)	Average bone ash	Average Ca per 100 cc. blood serum	Average P per 100 cc. blood serum
			sec.	ergs (×10^5)	quanta (×10^{14})	cc.	ergs (×10^5)	quanta (×10^{14})	gm.	per cent	per cent	mg.	mg.
II-AS-80	69	Sunshine carbon	8	339	459	43.4	754	1,021	342	100	35.15	8.21	8.02
IV-AHG	182	Mercury vapor	16	650	870	46.1	1,519	2,033	344	100	37.53	8.13	5.89
II-AS-80	70	Sunshine carbon	16	694	938	49.0	1,538	2,079	438	44	37.39	8.45	6.76
III-AMG-60	125	Magnesium "	8	796	1,105	62.0	2,031	2,820	462	56	38.04	9.64	5.26
I-AC-60	5	C carbon	8	989	1,323	63.7	2,409	3,223	562	16	41.30	9.89	6.70
IV-AHG	183	Mercury vapor	32	1,328	1,784	61.3	3,358	4,511	511	0	41.79	10.70	7.15
II-AS-80	71	Sunshine carbon	32	1,412	1,912	58.9	3,949	5,348	536	0	43.14	10.35	7.11
III-AMG-60	126	Magnesium "	16	1,548	2,150	65.6	3,859	5,360	602	0	43.25	11.85	5.39
III-AMG-45	123	" "	32	1,783	2,450	61.0	4,134	5,681	544	0	43.76	10.85	7.12
IV-AHG	184	Mercury vapor	48	1,938	2,598	62.8	4,761	6,383	513	0	43.19	10.90	7.16
II-AS-80	72	Sunshine carbon	48	2,112	2,862	57.7	4,909	6,652	531	0	42.97		6.85
I-AC-60	6	C carbon	16	2,016	2,676	65.8	5,423	7,199	582	0	43.94	11.20	7.64
III-AMG-80	130	Magnesium carbon	16	2,430	3,374	60.1	5,275	7,324	544	0	44.84	11.20	7.55
III-AMG-45	124	" "	48	2,759	3,790	60.9	6,220	8,544	571	0	44.14	10.85	7.32
III-AMG-60	127	" "	32	3,124	4,340	67.7	8,031	11,157	565	0	42.92	12.30	5.97
I-AC-80	7	C carbon	32	4,292	5,700	68.5	8,512	11,305	619	0	43.01	11.85	7.75
III-AMG-80	128	Magnesium carbon	48	4,740	6,588	71.1	9,924	13,793	602	0	42.35	12.35	6.22
I-AC-60	8	C carbon	48	6,780	9,012	67.1	16,754	22,270	603	0	42.90	11.50	7.61
III-AMG-80	132	Magnesium carbon	48	7,670	10,620	65.8	18,145	25,124	580	0	42.85	12.34	7.55
20L		Negative control; non-irradiated milk							270	100	34.51	8.99	6.73

$\times$ 10^3 ergs or more had been applied required 23.3 cc. or less, when tested by the standard assay method to give a +1 degree of calcification. Milk to which 989 $\times$ 10^3 ergs or less had been applied did not contain sufficient vitamin D to afford protection. Such milk has been shown to require from 26.7 to 45 cc. to give a +1 degree of calcification when tested by the standard method of assay. These comparative results are to be interpreted as showing a substantial degree of parallelism, within certain limits, between the results from the rat method, the rickets-preventing properties for chickens, and the amount of energy applied to the milk. A complete correlation between the amount of energy applied and the resulting antirachitic properties, determined by either method, cannot be extended to those milks to which the largest amounts of energy had been applied, because the inherent composition of milk limits the amount of vitamin D which may be formed by direct irradiation (5).

In order to evaluate in a more concrete manner the antirachitic properties of the different milks, Chart I has been constructed to show the average energy intake per day per chick per 100 gm. of body weight. The area defined by the lowest curve represents the range of energy intake from the five samples which did not contain sufficient vitamin D for complete protection. The area between the lowest curve and the second curve represents the range of energy intake which can be interpreted as permitting only marginal protection. The wide area defined by the marginal zone and the uppermost curve represents the range of energy intake from the fourteen different milks which supplied sufficient vitamin D to prevent rickets under the conditions of these tests.

These data may be used as a basis for calculating the vitamin D requirement of the growing chick if certain assumptions are permitted. In a previous paper (5) due notation was made of the assumptions which it was necessary to employ in arriving at a quantitative estimation of the amount of vitamin D in the irradiated milks used for these experiments. Such calculations show that milk (Sample 183) which had received the minimum energy necessary to prevent rickets contained 1.29 γ per liter. By reference to the food consumption records it was found that the average daily vitamin D intake was 0.0341 γ per chick per 100 gm. of body weight. The highest daily intake (0.0472 γ) was reached

during the 2nd week. After the 4th week the daily intake had declined to 0.029 γ and remained substantially at this level during the rest of the test period. The calculated vitamin D content of
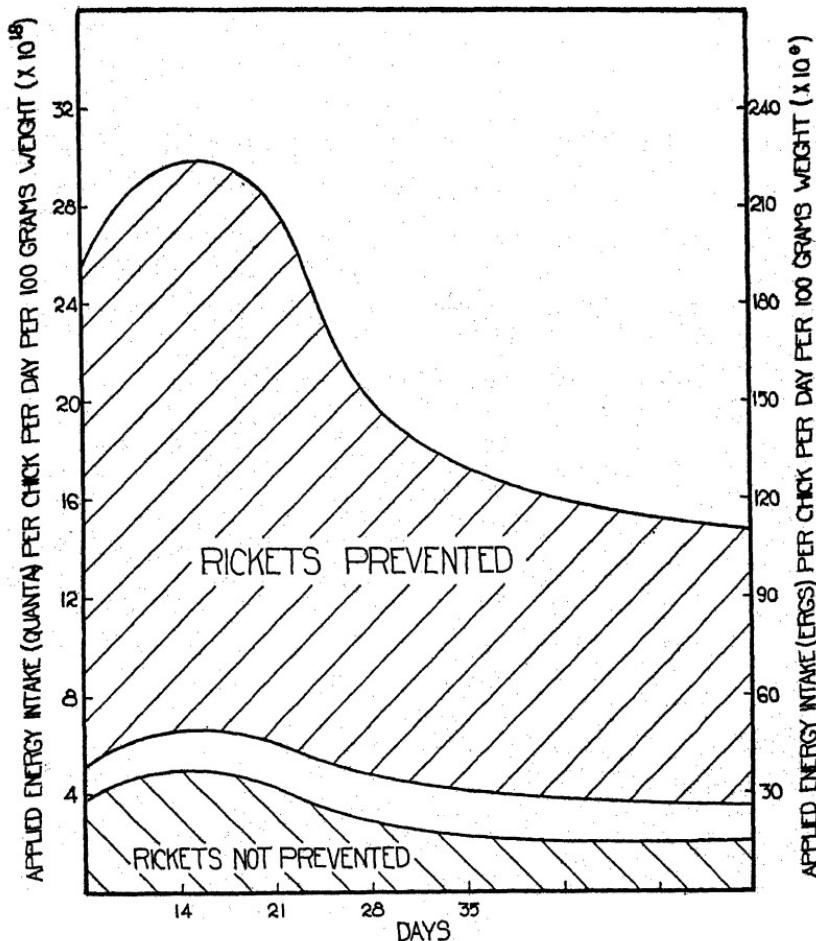

CHART I. The relationship between the quanta and ergs (2000 to 3000 Å.) applied to the milk consumed and the prevention of rickets in chickens.

Sample 5 was 1.12 γ per liter. This milk did not afford complete protection at an average daily intake of 0.0267 γ of vitamin D per chick per 100 gm. of body weight, the highest daily intake being 0.0346 γ during the 2nd week.

A further comparison of possible significance is the calculated vitamin D content of Sample 6, previously used for clinical studies (4). This milk contained 1.5 γ per liter and was found to protect colored infants fully from rickets during the winter months and was also shown to have definite curative value in a number of cases. Provided the feeding schedule called for 1 liter of the reconstituted dry milk per day for a 12 pound infant, the vitamin D intake would be approximately 0.027 γ per 100 gm. of body weight.

SUMMARY

1. The antirachitic properties of dry milk previously irradiated in fluid form by various carbon arcs and mercury vapor arcs may be accurately determined by the degree of protection against rickets afforded to growing chickens.

2. In order to prevent chickens from rickets under the conditions described, the minimum amount of energy between 2000 and 3000 Å. which must be applied per cc. of milk is approximately 1,328,000 ergs or 1784×10^{14} quanta. The vitamin D content of such milk was calculated to be 1.29 γ per liter.

3. A calculated daily average intake of 0.0341 γ of vitamin D per 100 gm. of body weight supplied as activated dry milk prevented rickets in chickens throughout the first 8 weeks of life. A calculated daily average intake of 0.0267 γ per 100 gm. of body weight failed to protect fully against rickets. A calculated daily average intake of vitamin D greater than 0.0341 γ per 100 gm. of body weight also prevented rickets, but superior physical condition, increased rate of growth, increase in bone ash, or abnormal relationships in the calcium and phosphorus content of the blood were not noted.

4. Within certain limits, there is a substantial degree of parallelism between the amount of energy applied to the milk and its antirachitic properties as determined by either the standard assay method with rats or its effectiveness in preventing rickets in chickens.

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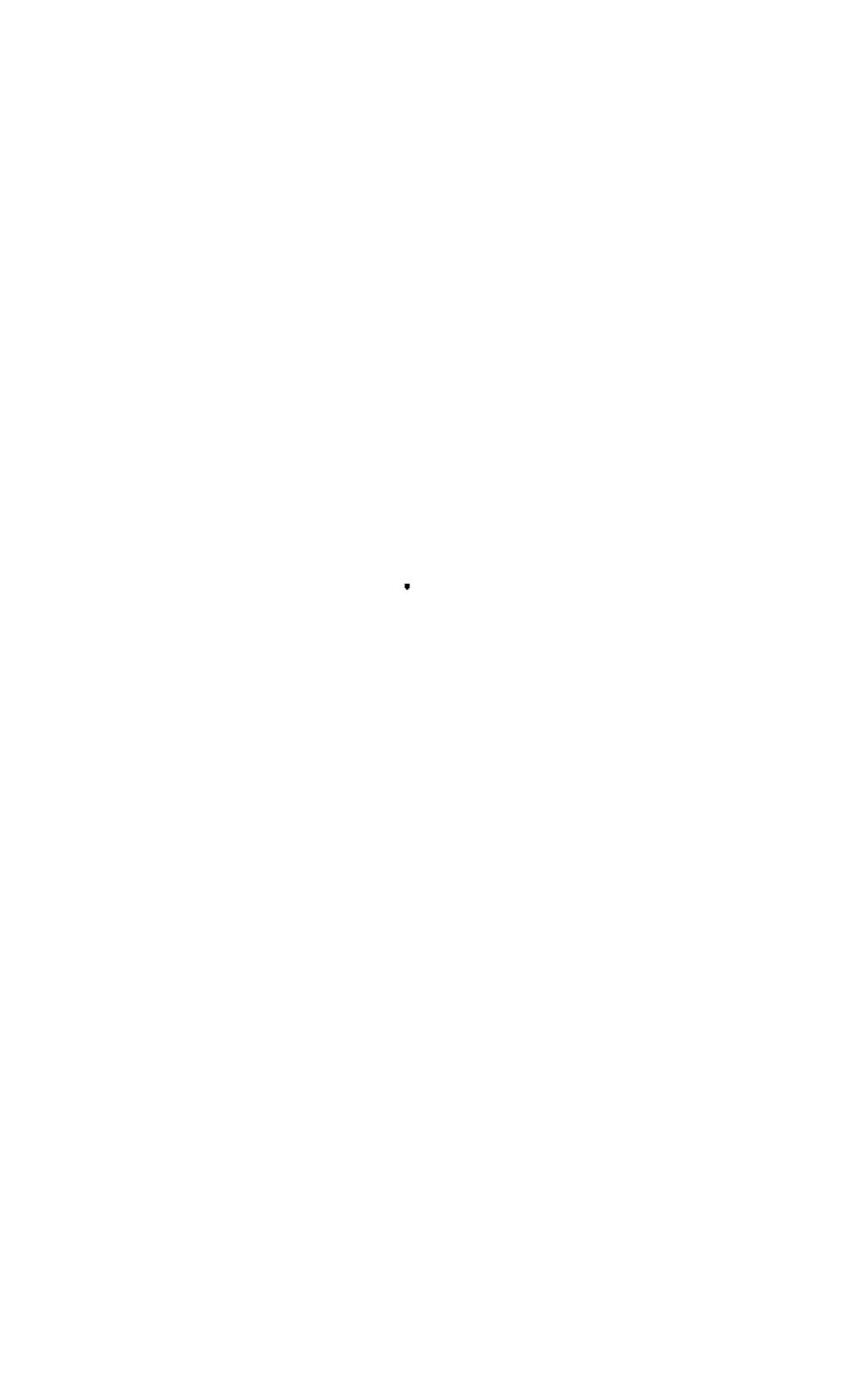
STUDIES IN VITAMIN A AVITAMINOSIS IN THE CHICK*

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The rat has been used almost exclusively as the laboratory animal in vitamin A studies. The results obtained with rats have been so consistent that little consideration has been given to the use of other animals. In fact the characteristic development of ophthalmia in the rat has led to considerable neglect in the study of other physiological changes occurring during vitamin A avitaminosis. Since chickens do not develop ophthalmia uniformly when deprived of vitamin A, investigations concerning the incidence of other symptoms during this dietary deficiency in the chick become necessary.

The early work on the fat-soluble vitamin requirements of chicks was reviewed by Emmett and Peacock (1) in 1923. From their own work they concluded that a very large percentage of the chicks fed a vitamin A- (vitamins A and D were not differentiated at that time) deficient diet developed symptoms of ophthalmia. Upon autopsy of their animals they found the presence of urates in the tubules, kidneys, and at times on the surface of the heart, liver, and spleen. Beach (2) had previously reported similar symptoms in a disease which he classed as poultry nutritional roup. Emmett and Peacock concluded that both conditions are the same and that poultry nutritional roup is due to the absence of the fat-soluble vitamins in the ration.

The following year Hart, Steenbock, Lepkovsky, and Halpin (3) reported their observations on the vitamin A requirement of chicks when the antirachitic factor was supplied in the form of ultra-violet light. They concluded as did Emmett and Peacock

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that growing chickens require vitamin A for normal development, but they failed to observe ophthalmia in the chicks suffering from vitamin A deficiency.

In 1927 Cruickshank, Hart, and Halpin (4) in their studies on the vitamin A and vitamin D content of cod liver meals described a typical vitamin A deficiency in chicks reared on a diet of 92 parts of white corn, 5 parts of yeast, 2 parts of $CaCO_3$, 1 part of NaCl, and skim milk *ad libitum*. The symptoms exhibited were: a staggering gait, a loss of control accompanied by emaciation and weakness, and a general ruffled condition of the feathers. They stated that the incidence of ophthalmia was very rare. Other workers (5, 6) have reported similar difficulties in producing ophthalmia in the chick. It is evident, therefore, that ophthalmia cannot be used as a criterion for the diagnosis of vitamin A deficiency in this species.

Cruickshank, Hart, and Halpin found kidney lesions in practically all of the chickens that died during the experiment. The kidneys were pale in color due to the accumulation of large amounts of urates in these organs. The ureters were distended and filled with urates. Some of the birds killed at the termination of the experiment, although in a very weak condition, displayed only slight kidney lesions. Cruickshank, Hart, and Halpin made no attempt to follow the uric acid content of the blood. The addition of 2 per cent cod liver oil to the basal ration prevented this condition and allowed the development of normal chicks.

Hughes, Lienhardt, and Aubel (7) reported that pigs, chickens, and cows, kept on vitamin A-deficient diets, developed, without exception, a marked nervous disorder characterized by blindness, incoordination, and spasms. Histological examinations of nerve tissue from the pig showed a degeneration of the nerve bundles of the spinal cord, optic, sciatic, and femoral nerves. Although they reported no histological examinations of the chicken, they suggested that the nerve degeneration in this animal is similar to that found in the pig.

Seifried (8) made extensive histological studies on chicks suffering from vitamin A avitaminosis but he did not study the nerve changes. He states, however, that there is a general keratinization of all parts of the respiratory tract, the olfactory region, and the

upper alimentary tract, especially the mucous glands and their ducts.

Capper, McKibbin, and Prentice (9) used the chicken to demonstrate that the fowl as well as the rat can convert carotene into vitamin A. They used chickens which were intended for another experiment and the development of vitamin A deficiency was unexpected since the basal ration contained some yellow corn. The chickens were approximately 16 weeks old before a deficiency developed. These workers described the symptoms in chickens as follows: "Their sense of balance appeared to be disturbed and they walked with difficulty and with a staggering gait." Deposits of urates were found around the heart, liver, and other organs, and the disease was diagnosed as visceral gout. The livers were found to contain no vitamin A. Cod liver oil concentrates prevented or cured this condition.

A survey of these early papers on the relation of vitamin A to the nutrition of the chick has brought to mind a number of questions, which we have attempted to answer by the work reported in this paper. Are the symptoms described by Cruickshank, Hart, and Halpin, and Capper, McKibbin, and Prentice, specific for vitamin A deficiency, and can conditions be standardized sufficiently to use the chick for vitamin A studies? Does the uric acid content of the blood increase during vitamin A deficiency, and are the symptoms reported dependent upon these blood changes? Is the uric acid content of the blood dependent upon kidney degeneration?

EXPERIMENTAL

Day old white Leghorn chicks were used for all the experimental work reported in this paper. However, we have used chickens taken from the general poultry flock when 6 weeks old with equal success. These older birds developed typical symptoms of vitamin A deficiency in a period of 3 weeks when placed on a vitamin A-low ration. In our earlier work we used a ration consisting of 97 parts of white corn, 2 parts of $CaCO_3$, 1 part of NaCl, and skim milk *ad libitum*, together with irradiation, for the production of vitamin A deficiency. Chicks placed on this diet when 1 day of age failed to grow very well and died before typical symptoms developed and before they were old enough to supply

sufficient blood for analysis. Hart, Steenbock, Lepkovsky, and Halpin (3) obtained considerable growth on this ration in the presence of sunlight, but their chicks were kept on shavings rather than on screens. This ration also requires the use of liquid milk, and is undoubtedly somewhat deficient in the B vitamins.

The ration used by Hart, Kline, and Keenan (10) for the production of rickets in chicks under standardized conditions has been found so successful that we attempted to modify this ration sufficiently to make a standard diet for the production of vitamin A deficiency. The yellow corn was replaced by white corn, the yeast was increased to 2 per cent, and the chicks were irradiated to supply vitamin D. The basal ration was compounded in the following proportions.

> 58 parts ground white corn
> 25 "   wheat middlings (standard)
> 12 "   crude casein
> 1 part common salt
> 1 "   precipitated calcium carbonate
> 1 "   "   "   phosphate
> 2 parts dried yeast (Northwestern)

The corn, middlings, and casein were mixed in relatively large quantities, but the salts and yeast were added weekly to smaller portions. The chicks were given this ration, together with water *ad libitum*, and were irradiated 20 minutes three times weekly. They were kept in small pens in our animal room where there is no direct sunlight. The pens were equipped with wire screen floors and small electrically heated brooders.

When day old chicks were placed on this ration they grew at a fairly normal rate for a period of 3 weeks. Between the 3rd and 4th weeks growth ceased and often there was a decided decline in weight during the 4th week. The affected birds exhibited a staggering gait and a general incoordination of their movements. As the condition progressed they appeared very drowsy and moved only when disturbed. There was considerable tendency for them to crouch on their haunches in order to maintain their equilibrium. In advanced stages the animal lay on one side, and the head fell forward. The feathers were very ruffled and there was some soreness around the eyes, but there was no typical ophthalmia. The beak and shanks were extremely colorless. There was no decided

decrease in appetite and often the animals made considerable effort to reach the feed even when in a very weakened condition. Chicks in the same group became affected with extreme unformity. These conditions lasted for only a few days, when death ensued. Most of the birds were dead before the end of the 5th week, while many of them succumbed before the end of the 4th week. Every chick which we have placed on this diet has developed, without exception, these specific symptoms.

The growth curves for three chicks fed the basal ration are plotted in Chart I. The growth curves and the symptoms exhibited by these birds are typical of all chicks fed this ration. The records of two chicks reared on the same ration plus 2 per cent cod liver oil are included for comparison. The excellent growth and normal development of these chicks demonstrates that the basal ration furnishes other factors in sufficient quantity.

Autopsies were performed on all the animals that died or were killed for samples of blood. Especial attention was given to the accumulation of urates in the kidneys and renal tubules. In many cases the tubules were greatly distended due to the accumulation of urates. The kidneys were also filled with urates, in some cases to such an extent that these organs appeared almost white. However, in a number of cases the chicks would exhibit incoordination when there was no evident accumulation of urates in the urinary tract.

The uric acid content of the blood of a large number of vitamin A-deficient and normal chicks was determined, but the results from only one series of experiments will be given in detail. 80 chicks were divided into eight groups of ten chicks each and fed as follows: Group 1, basal ration; Group 2, basal ration plus 2 per cent cod liver oil; Group 3, basal ration prepared with corn, middlings, and casein which had been heated at 95° for 72 hours; Group 4, same as Group 3 plus 2 per cent cod liver oil; Group 5, basal ration in which the corn was decreased to 52 parts and the casein increased to 18 parts; Group 6, same as Group 5 plus 2 per cent cod liver oil; Group 7, basal ration in which the corn was decreased to 46 parts and the casein increased to 24 parts; Group 8, same as Group 7 plus 2 per cent cod liver oil. This arrangement not only allowed us to study the uric acid content of the blood of vitamin A-deficient and normal chicks, but gave us an oppor-

tunity to determine the influence of the protein intake on the distribution of uric acid in the blood. Groups 3 and 4 were included to determine whether heating would decrease any of the remaining vitamin A in the ration sufficiently to alter the usual results.
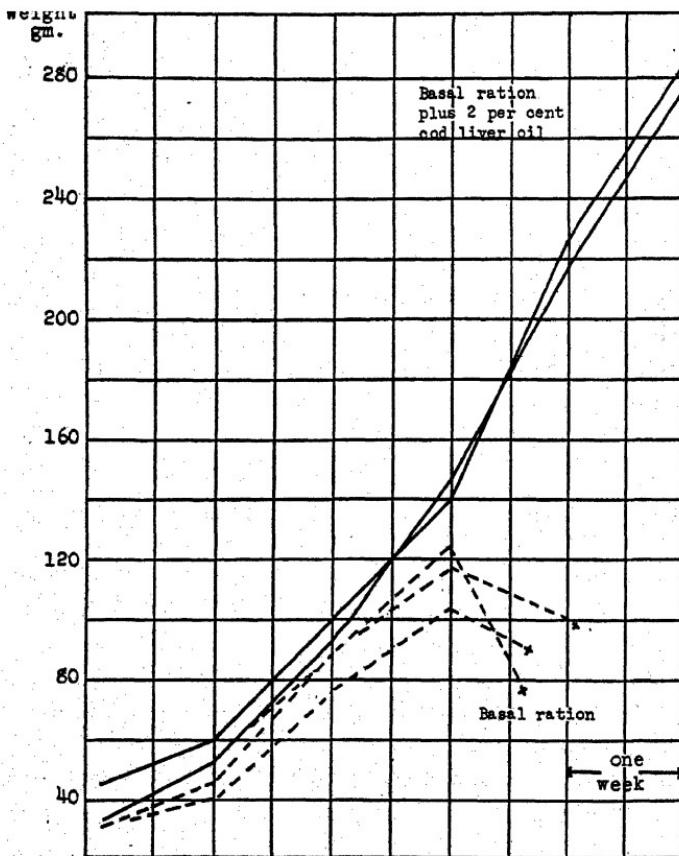

CHART I. Growth records of three chicks fed the basal ration alone and two chicks fed the basal ration plus 2 per cent cod liver oil.

All the chicks placed on the basal or the modified basal ration developed the typical symptoms of vitamin A deficiency in the usual length of time. The chicks fed the heated ration did not develop the symptoms at a faster rate, which demonstrates that the basal ration must be very low in vitamin A. The chicks on
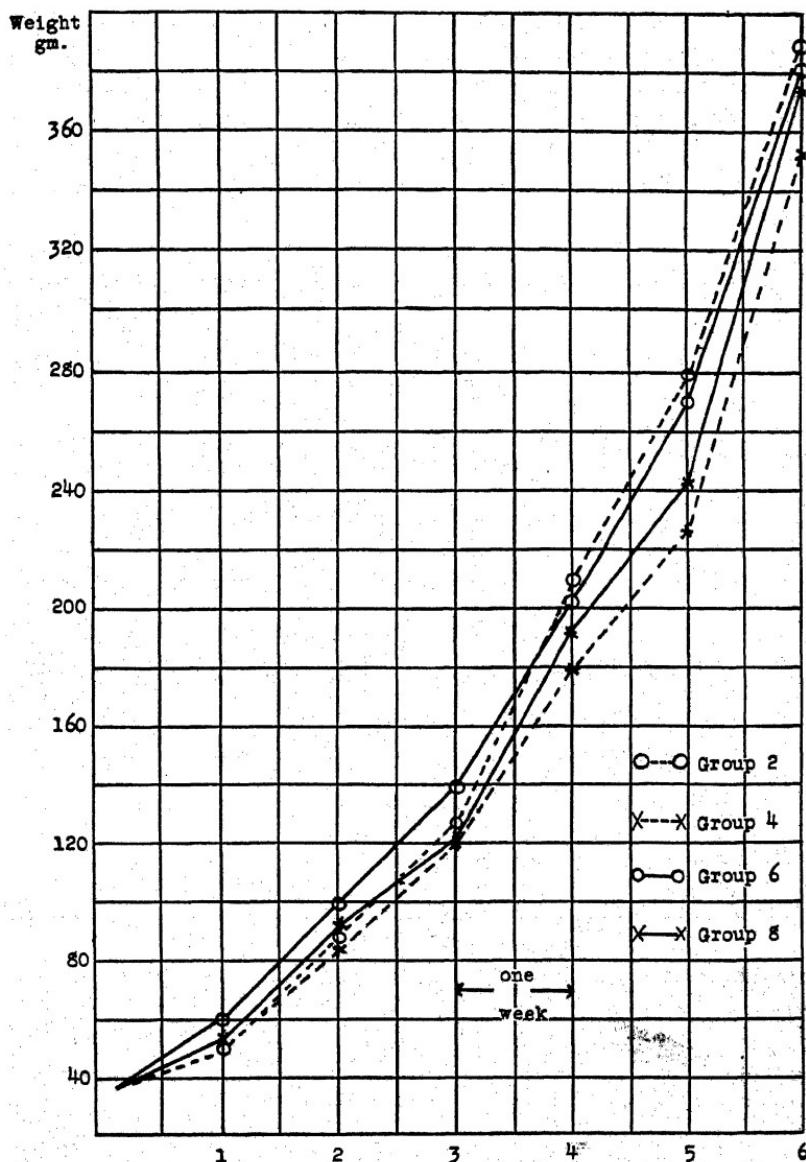
CHART II. Average growth records of the chickens in Groups 2, 4, 6, and 8. All groups received 2 per cent cod liver oil. Group 2 received the basal ration which contained 12 parts of casein; Group 4, the same ration heated at 95° for 72 hours; Group 6, the basal ration which contained 18 parts of casein; and Group 8, the basal ration which contained 24 parts of casein.

the high protein diet did not differ in their reactions from those on the unmodified basal ration.

The average growth curves for the chicks in the groups receiving cod liver oil are plotted in Chart II. It is readily seen that the changes in the basal ration had little effect on the growth of the chicks in the presence of sufficient vitamin A. The growth of

TABLE I

Uric Acid Content of Blood from Normal Chicks (Figures Obtained from 53 Animals)

Age	Uric acid per 100 cc. whole blood		
	Minimum	Maximum	Average
wks.	*mg.*	*mg.*	*mg.*
3– 6	4.6	5.9	5.2
6– 9	3.8	6.6	5.1
9–12	4.4	5.4	4.9
12–15	3.7	6.8	5.9

TABLE II

Effect of Protein Intake on Uric Acid Content of Blood of Normal Chick

Group No.	Ration	Uric acid per 100 cc. whole blood
		mg.
2	12 parts casein	4.8
4	12 " " (heated)	4.9
6	18 " "	4.8
8	24 " "	5.4

the animals on the heated ration was slightly less than the other groups and the early increment in the weights of the birds receiving 18 per cent casein was the greatest, although the increase for the chicks on the lowest level of casein was equally as good after the 3rd week.

The blood for analysis was obtained from the external jugular, and the uric acid content was determined by Benedict's method (11). In Table I are shown the average figures for normal chicks, produced on the basal ration plus 2 per cent cod liver oil. The results indicate that the average figure for chicks up to 12 weeks

of age is 5.0 mg. per 100 cc. of blood. There is a tendency for the uric acid to increase slightly with age.

The effect of the protein intake on the uric acid content of blood in the presence of sufficient vitamin A is given in Table II. Again the average figures check very well with the normal of 5 mg. per

TABLE III

Uric Acid Content of Blood Together with Observations on Kidney Change and Incoordination in Chicks on Vitamin A-Deficient Ration

Age of chick	Uric acid per 100 cc. whole blood	Eye	Loco-motion	Kidney and ureters	Age of chick	Uric acid per 100 cc. whole blood	Eye	Loco-motion	Kidney and ureters
days	*mg.*				*days*	*mg.*			
21	5.8	−	++	+	34	22.5	+	+	++++
25	8.7	−	++	−	36	6.7	+	+	+
25	11.8	−	+	+	36	6.9	−	++	+
26	4.8	−	+	++	36	6.9	−	++	+
26	9.8	−	++	+++	36	7.1	−	++	+
26	30.2	+	++	++++	36	8.6	−	+	+
27	4.4	+	+	−	36	9.7	−	+	++++
27	4.8	−	+	+	41	44.4	+	++	++++
27	5.1	−	+	−	43	8.1	−	+	+++
27	7.1	+	+	−	43	9.3	−	++	++
27	24.6	+	+	++++	47	7.1	+	+	+++
28	7.7	−	+	−	47	11.3	+	++	+
29	11.5	−	+	+++	48	25.3	+	++	++++
32	5.4	−	+	−	48	29.6	+	++	++++
32	6.7	−	+	−	59	7.8	−	+	+
32	7.2	−	+	++	60	37.0	+	++	++++

Eye. − indicates normal; +, some soreness.

Locomotion. − indicates normal; +, difficulty in walking; ++, unable to stand.

Kidney and Ureters. − indicates no abnormal amount of urates; +, small amount; ++, large amount; +++, very large amount; ++++, kidneys impaired and ureters distended with urates.

100 cc. The figure for the chicks on the highest casein level is slightly higher but probably not enough to be significant. In the case of the vitamin A-deficient chicks, the uric acid content of the blood was found to be more closely related to factors other than the protein intake.

In Table III we have given the uric acid values for a number of

individual vitamin A-deficient chicks. The gross symptoms and autopsy findings for each animal are also included. It is evident that a number of interesting conclusions can be drawn from these results. The uric acid figures vary from the normal (5 mg. per 100 cc. of blood) to values as high as 44 mg. per 100 cc. of blood. The uric acid values cannot be correlated with the age of the chick. There is a tendency for the uric acid values to become greater as the degree of incoordination progresses. However, a number of the chickens exhibited great difficulty in locomotion before the uric acid value had increased much above normal, which indicates that the incoordination noted is not due to an increased uric acid content of the blood.

It is more likely that the incoordination observed is due to nerve degeneration. Hughes *et al.* (7) have shown that this is the case in the pig, and Mellanby (12) has recently reported that young puppies kept on vitamin A-deficient diets exhibit degenerative changes in the spinal cord in the form of demyelination of the nerve fibers. Studies on the cord changes in the chick are now in progress.

The high uric acid content of the blood of a number of the chickens indicated that the normal elimination of uric acid is disturbed during vitamin A deficiency. A general correlation was noted between the uric acid content of the blood and the degree of kidney hypertrophy. This led us to make a histological comparison of the kidneys of normal and vitamin A-deficient chicks. Whole kidneys were removed carefully from the surrounding tissue in each chick, after autopsy, and fixed immediately in Bouin's fluid. After fixation the tissue was hardened in graded alcohol, cleared, and embedded in paraffin. Cross-sections from each lobe of individual kidneys were mounted and stained with iron-hematoxylin-eosin.

Sections from the kidneys of chicks exhibiting extreme vitamin A deficiency showed in every case a slight nephrosis and an occasional parenchymatous degeneration. In most cases the degeneration was in the proximal convoluted tubules, while the distal portion of the collecting tubules and ducts of Bellini had undergone considerable dilatation. The cytoplasm of the cells in the proximal part of the tubule was clear in the basal portion while the granular material was massed in the periphery or distal portion

of the cell. The basal portion often contained fat globules. Some of the tubules contained leucocytes, giant cells, and cellular débris; others, considerable colloidal material. Practically all the sections showed hyaline deposits in both arteries and glomeruli. Kidneys from chicks with large amounts of uric acid in the blood showed the most marked degeneration.

These results demonstrate that definite pathological changes occur during vitamin A deficiency, which suggests that the uric acid metabolism is not disturbed, but that the structure of the kidney is damaged sufficiently to prevent the normal elimination of uric acid. In this respect it is interesting to refer to the work of McCarrison (13) on the relation of vitamin A deficiency to urinary calculi. He reports the incidence of renal calculi in 22 per cent of the rats raised on diets compounded from food in common use by the people of India. This condition was prevented by the addition of vitamin A. He states that no tissue suffers more severely from want of vitamin A than that of the urinary tract and suggests that the keratinized epithelium from this tract may form the nidus around which calculus deposition occurs.

Since birds make no attempt to destroy uric acid and also convert the greater part of their urea to uric acid for elimination, the uric acid content of the blood of this species is more readily affected by degenerative changes in the kidney than the blood of other animals. In the rat the amount of uric acid excreted is very small. This fact undoubtedly explains the results reported by Osborne and Mendel (14) and van Leersum (15); namely, that the urinary calculi observed in rats on a vitamin A-deficient diet consist chiefly of calcium salts.

Man occupies an intermediate position between birds and reptiles and mammals other than man in regard to the destruction of uric acid. The changes observed in the uric acid elimination in the chick during vitamin A deficiency, therefore, are probably more applicable to man than those observed in other animals.

SUMMARY

1. A standard ration for the production of vitamin A avitaminosis in the chick is described.

2. Growth curves for chicks reared on this ration alone and on the basal ration plus 2 per cent cod liver oil are given.

3. The typical symptoms of vitamin A deficiency in the chick are described.

4. The uric acid content of the blood of normal chicks is approximately 5.0 mg. per 100 cc. of whole blood. The amount of uric acid in the blood is independent of the protein intake.

5. The uric acid content of the blood of vitamin A-deficient chicks varies from normal values to amounts as high as 44 mg. per 100 cc. of blood.

6. The kidney undergoes definite pathological changes during severe vitamin A avitaminosis. The amount of uric acid in the blood is dependent upon the degree of kidney damage. The degree of incoordination is independent of the uric acid content of the blood.

7. Vitamin A deficiency does not disturb uric acid metabolism, but does injure the structure of the kidney sufficiently to prevent normal elimination of uric acid.

We are indebted to Mr. Paul H. Phillips for assistance in the examination of the histological sections.

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CAROTENE AND XANTHOPHYLL AS SOURCES OF VITAMIN A FOR THE GROWING CHICK*

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The observation that the occurrence of the yellow plant pigment and the growth-promoting property attributed to the presence of fat-soluble vitamin A seemed to be intimately associated in green plant tissue, yellow maize, and some roots, was made by Steenbock and Boutwell (1) in 1920. Further, these investigators (2) demonstrated that the vitamin was concentrated in the carotene-rich fraction extracted from alfalfa.

The more recent findings of von Euler, von Euler, and Hellström (3), that carotene itself possesses vitamin A activity, have been substantiated by a number of workers (4–7). In all of these experiments rats were used for testing the curative effect of carotene in vitamin A avitaminosis. Moore (8) also used rats for demonstrating that carotene is converted into vitamin A by the animal body. Similar studies, conducted on other animals, have been made in only a few cases. Capper, McKibbin, and Prentice, (9) concluded that chicks could be reared to maturity on a synthetic vitamin A-free diet to which carotene was added, and that the carotene was converted into vitamin A. However, since the chicks used in this work were not placed on the vitamin A-deficient diet until they were 6 weeks old, it is difficult to draw conclusions regarding the value of carotene during the early growing period. Karrer, von Euler, and Rydbom (10) have presented data showing the effect of xanthophyll and carotene when fed to chicks. They used a vitamin A-free ration consisting mainly of rice starch, casein, and salt mixture, with the proper

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addition of irradiated peanut oil, yeast, and marmite to complete the vitamin requirement. The chicks placed on this ration when 10 days of age survived for 3 to 4 weeks and made a gain in weight of 25 to 50 gm. in that period. When the ration was supplemented with 0.03 mg. of xanthophyll, a gain of less than 50 gm. was obtained by the end of the 3rd week, when death occurred. In the case of a carotene supplement, 0.03 mg. daily caused an increase in weight of only 75 to 100 gm. in 6 weeks. No data are given to show whether or not good growth might be obtained on the experimental ration used, when the chicks were supplied with sufficient vitamin A in the form of cod liver oil. It is difficult to evaluate the results obtained with carotene feeding where the growth increments are so small.

The conclusion made by these workers that xanthophyll has a toxic effect when fed to chicks is not substantiated by a study of the growth curves presented. It is difficult to understand why such a conclusion should be made from the small differences obtained between the rates of growth and the length of survival of the xanthophyll-fed group and basal group.

In view of the above results it was decided to reinvestigate the value of carotene and xanthophyll as a source of vitamin A for chickens. The basal ration used was one which gave good growth when sufficient vitamin A was supplied in the form of cod liver oil. Chicks on this ration, which was made up largely of natural grains, grew to a weight of 375 gm. in a period of 6 weeks. Also, the ration without the cod liver oil supplement caused development of vitamin A deficiency symptoms during the 4th week, with death usually following in 4 to 6 days.

EXPERIMENTAL

The carotenoids used to supplement the basal ration were isolated from spinach. The fresh leaves were dried at 37° and extracted with acetone, according to the method outlined by Schertz (11). The crude xanthophyll was separated from the carotene by extraction with methyl alcohol. The xanthophyll was recrystallized five times from methyl alcohol, giving a crystalline product with a melting point of 174° (uncorrected).

The carotene preparation used was obtained from the same acetone extract, and recrystallized three times from carbon

disulfide with 95 per cent ethyl alcohol. The melting point of this crystalline product was 172.5° (uncorrected). Spectrographic analysis of the products by means of the small Zeiss narrow dispersion spectroscope, gave absorption bands at 478 to 465, 450 to 436, and at 420 $m\mu$, for xanthophyll, in methyl alcohol, while the carotene preparation in carbon disulfide gave bands at 515 to 505, 490 to 480, and at 450 $m\mu$.

For feeding purposes the carotenoids were dissolved in Wesson oil (a cottonseed oil) at such a concentration that 0.5 cc. of oil contained the amount of pigment to be given daily. In this way the oil intake of all the birds was kept uniform. The oil was fed directly to each chick daily by means of a graduated pipette.

Day old white Leghorn chicks, weighing from 30 to 35 gm., were used for all the work. They were divided into groups of nine, and placed in suitable warmers heated by incandescent lamps, in a semidarkened room. The cages used were provided with screen bottoms.

The same basal ration was used as is outlined in the previous paper by Elvehjem and Neu (12). It consisted of:

	per cent
White corn	58
Standard wheat middlings	25
Crude casein	12
NaCl	1
Dry yeast (Northwestern)	2
Precipitated $CaCO_3$	1
" $Ca_3(PO_4)_2$	1

The experimental plan used included a prophylactic type and a curative type of feeding.

In the prophylactic feeding experiment the groups were fed as follows:

Group 1, basal ration only
2,   plus 0.5 cc. cod liver oil daily
3,   0.5 " Wesson " "
4,   0.05 mg. xanthophyll "
5,   0.25 " " "
6,   0.03 " carotene "

In the curative feeding experiment the chicks were first brought down with symptoms of vitamin A deficiency, and supplementary

feeding of carotene and xanthophyll was started on the 25th day. The rations used were as follows:

```
Group 7, basal ration plus 0.10 mg. xanthophyll daily
  "    8,    "       "      "  0.25  "        "        "
  "    9,    "       "      "  0.05  "  carotene       "
```

Vitamin D was supplied to all birds, except those in Group 2 which received cod liver oil, by direct irradiation with a quartz mercury lamp, 10 minutes daily at a distance of 24 inches. When cod liver oil was fed the intake was so adjusted by dilution with Wesson oil, that it constituted 2 per cent of the ration consumed. The supplementary oil feeding in Groups 1 to 6, in which the prophylactic technique was used, was postponed until the 2nd week in order to avoid an oil intolerance at the early age. During the 2nd week one-half the daily dose was administered, and the full dose was given thereafter. In Groups 7, 8, and 9, in which the curative technique was employed, the oil feeding was not started until the 25th day. At this time there was an indication of vitamin A deficiency in the majority of chicks, judging from the external symptoms, such as lack of muscular coordination, and ruffled feathers, and also by a reduced rate of growth, or even loss in weight.

It will be seen from a study of Chart I that good growth was obtained in all groups up to the 4th week. At this time differences in the experimental groups became evident, both in weight and in gross appearances. The chicks in Group 1, receiving the vitamin A-free basal ration, developed severe symptoms of vitamin A deficiency during the 4th week, and death followed 6 to 8 days later. The external symptoms of vitamin A deficiency noted in the chick are a dry and ruffled appearance of the feathers, a staggering gait, or even an inability to use the legs. In the early stages the chick will often fall backward when startled, showing complete incoordination of movement. In addition to these symptoms an examination of the internal organs invariably revealed an accumulation of a white deposit of urates in the ureters and kidneys, a condition which is described in detail in the preceding paper by Elvehjem and Neu (12). All birds in Group 1, examined after death, showed lesions in the kidneys, and often a

disintegration of that organ, with dilatation of the ureters caused by an accumulation of urates.
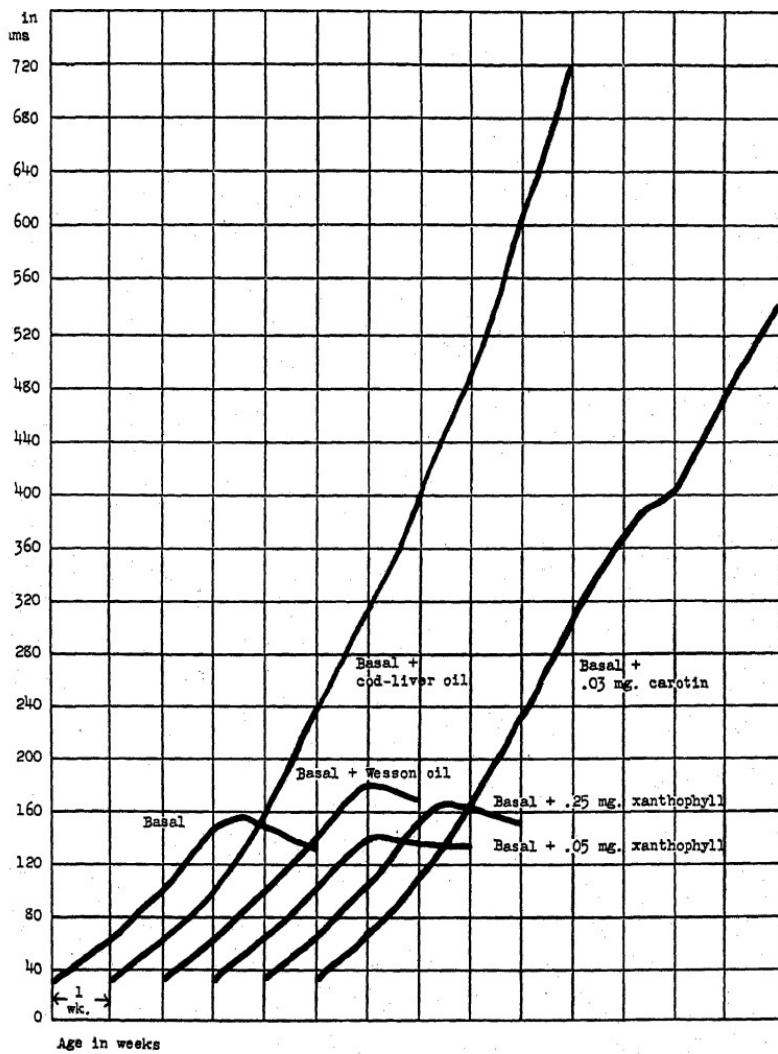

CHART I. These curves represent the average growth records of the chicks in Groups 1 to 6 from the prophylactic feeding experiment.

The records for the birds of Group 2, which received sufficient vitamin A as cod liver oil, demonstrate the value of the basal

ration used for obtaining good growth. In this group an average
weight of 395 gm. was obtained at 6 weeks. In Group 3, the
Wesson oil-fed group, vitamin A avitaminosis symptoms occurred
at the same time as in Group 1, followed by death of the chicks,
even though a slightly better growth was obtained. The typical
internal condition of the accumulation of large amounts of urates
in the ureters and on the surface of the kidneys was noticed on
autopsy. The severity of the symptoms indicated the lack of
any appreciable amount of vitamin A in the Wesson oil used.


FIG. 1   FIG. 2

FIG. 1. Chick from Group 6 weighing 320 gm. at 5 weeks. This chick
received 0.03 mg. of carotene daily. It exhibited no vitamin A deficiency.
FIG. 2. Chick from Group 5, weighing 160 gm. at 5 weeks. This chick
received 0.25 mg. of xanthophyll daily. It developed symptoms charac-
teristic of vitamin A deficiency.

In Groups 4 and 5, where xanthophyll was administered prophy-
lactically, the conditions obtaining were exactly like those of the
basal group. Here again the condition of the kidney and ureters
noted on autopsy was typical. The slightly better growth with
the higher level of xanthophyll is not significant, since the vitamin
A deficiency symptoms were alike in both groups. In Group 6,
where carotene was used at a level of 0.03 mg. daily, the chicks
grew as well as those receiving cod liver oil, and appeared normal
in every respect, until the 7th week. See Figs. 1 and 2. At this

time the rate of growth was retarded, and a few of the chicks showed slight incoordination of their movements.

When the curative feeding technique was used, we found again the lack of any effect with xanthophyll at levels of 0.10 and 0.25 mg. daily. These data are summarized in Chart II. The carotene addition, however, had a phenomenal effect both on the growth and the appearance of the birds. The effect is well described by Mellanby (13) when he makes the statement that there
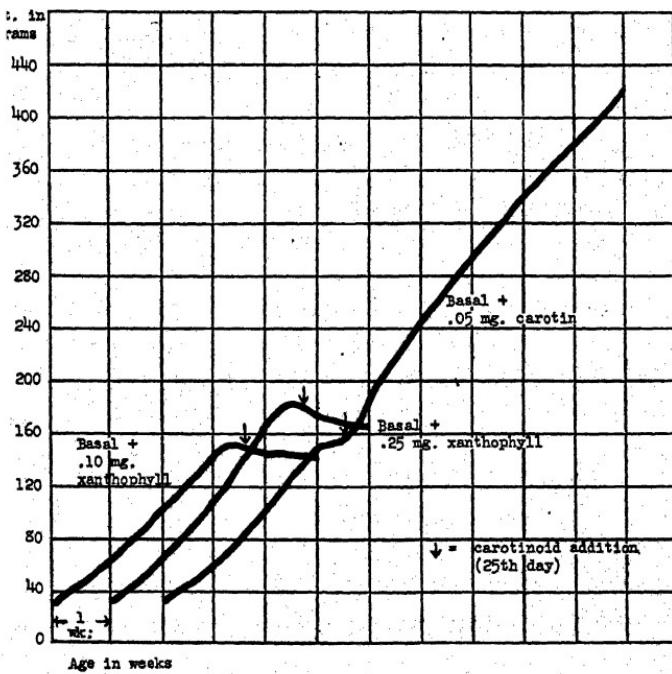

CHART II. These curves show the average growth records of the chicks in Groups 7, 8, and 9 from the curative feeding experiment.

are few more striking effects in animal nutrition than the improvement brought about by the daily addition of carotene to an animal suffering from such a deficiency.

Here again, however, there is evidence that, as the weight of the birds approached 400 gm. in the 8th week, the amount of carotene administered, 0.05 mg. daily, was insufficient. Upon terminating the experiment at 9 weeks, the birds were killed and examined for the presence of urates. In both groups receiving

the carotene, Groups 6 and 9, the internal organs of the birds were found to be normal, with two exceptions where there were a few small lesions in the kidneys. Our results indicate that the amount of carotene necessary to prevent the appearance of vitamin A avitaminosis symptoms is dependent upon the weight of the chick, 0.03 mg. daily being sufficient for the first 7 weeks, or for birds weighing up to 400 gm. Where curative feeding is used the requirement is slightly greater. Capper *et al.* (9) found it necessary to use from 5 to 10 mg. of carotene daily for chickens weighing up to 2000 gm., when they had been previously brought down with a vitamin A deficiency.

The work of Palmer and Kempster (14) indicates that the yellow pigment in the legs and beaks of chicks can be increased by xanthophyll feeding. In no case, in our experiments, where xanthophyll was fed, did the legs and beaks of the chicks show more than a faint increase in yellow pigment. The level of carotenoid fed by Palmer and Kempster, however, was undoubtedly much higher than that used in our experiments. There is no evidence in our work that xanthophyll exhibits toxic properties when fed to chicks. The condition developed in the xanthophyll groups was exactly like that of the basal group, a fact contrary to the report of Karrer, von Euler, and Rydbom. This was true even though the level of xanthophyll used in our experiments was 8 times that used by Karrer and associates.

The fact, then, that xanthophyll (m.p. 174°) prepared from spinach cannot serve as a source of vitamin A in the growth of the chick further emphasizes the specificity of carotene as a source of vitamin A.

CONCLUSIONS

1. Xanthophyll, m.p. 174°, prepared from spinach does not serve as a source of vitamin A in the chick. There is no evidence of toxic effects, even when fed at levels of 0.25 mg. daily per chick.

2. Carotene, m.p. 172.5°, prepared from spinach, when fed in adequate amounts, may serve as a source of vitamin A for the chick.

3. When the chicks reach the age of 7 to 8 weeks, 0.03 mg. of carotene daily is not sufficient when it is the sole source of vitamin A.

4. Chicks that have been depleted of vitamin A require more than 0.05 mg. of carotene daily in order to grow to maturity.

5. The basal ration used in this experiment, when fed to chicks, is excellently suited for the production of vitamin A deficiency in 3 to 4 weeks. When supplemented with vitamin A, however, good growth and normal development of chicks is obtained.

Note—While this report was being prepared, there appeared a note by Russel and Weber (15) on the physiological action of plant pigments in vitamin A deficiency. The results as to the action of carotene and xanthophyll are in complete agreement with the findings of our experiments.

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STUDIES ON CRYSTALLINE INSULIN*

XIV. THE ISOLATION OF GLUTAMIC ACID

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INTRODUCTION

Previous investigations on the chemical composition of insulin have shown that the following amino acids are present in the insulin molecule: cystine (about 12 per cent), tyrosine (about 12 per cent) (1); arginine, histidine, lysine, and leucine (2). It was estimated from analytical data and from the yields actually obtained that approximately 40 per cent of the molecule was accounted for by these amino acids. It is obvious that this figure represents too low rather than too high an estimate since great losses are unavoidable in the separation of amino acids obtained from the few gm. of insulin protein hydrolyzed. But even if allowance is made for this, it seemed that a sufficiently large part of the molecule had remained unexplored to justify a new inquiry into its composition. Moreover, there was still some hope of encountering in this search a constituent different in its composition from that of an amino acid, and which could perhaps be connected with the physiological action of the hormone. A new hydrolysis experiment was therefore undertaken on 4 gm. of crystalline insulin. Special attention was given to the dicarboxylic acid fraction, since the fairly high percentage of ammonia nitrogen (9.58 per cent) found in the determination of the nitrogen distribution (3) indicated the presence of this group of amino acids. We have been able to isolate glutamic acid from the hydrolysate, but have obtained no evidence for the presence of

aspartic acid or hydroxyglutamic acid. It therefore seems reasonable to assume that glutamic acid constitutes most, if not all, of the dibasic amino acid fraction. The occurrence of other amino acids besides those mentioned above could not be ascertained, though we obtained small quantities of material in fractions which should contain proline and oxyvaline. Leucine was found in considerable amounts and seems to constitute a large part of the molecule (25 to 30 per cent). Taking into account all the evidence gained in hydrolysis experiments on crystalline insulin, we feel justified in the statement that the insulin molecule is built up of comparatively few amino acids and that, in all probability, it does not contain constituents which are essentially different in their nature from amino acids. In the following we shall give only a brief outline of the manner in which the glutamic acid fraction was obtained. The work on the various other fractions obtained in the hydrolysis of insulin will be reported separately.

<div align="center">EXPERIMENTAL</div>

Insulin (4 gm.) was boiled with 40 cc. of 25 per cent sulfuric acid for 25 hours. After neutralization with barium hydroxide, the filtrate from the barium sulfate was concentrated, *in vacuo*, to about 20 cc. and freed by filtration from the cystine and tyrosine which had precipitated out during the evaporation. The filtrate was extracted for 30 hours with butyl alcohol according to Dakin (4) in an especially constructed all-glass extraction apparatus. The aqueous solution (after extraction with butyl alcohol for 30 hours) containing the dibasic acids and the hexone bases was filtered from cystine which was thrown out of solution during the extraction. The hexone bases were separated from the dibasic amino acids by phosphotungstic acid. After removal of the excess of phosphotungstic acid in the filtrate with barium hydroxide the solution was neutralized with sulfuric acid and evaporated nearly to dryness *in vacuo*. The residue was taken up in barium hydroxide solution and an excess of alcohol was added to the filtrate. After standing in the ice box for 3 days the precipitate was filtered off (centrifugation is more convenient), dissolved in water, and the barium salts reprecipitated with alcohol. It was found that the precipitate was contaminated with cystine and smaller amounts of histidine and arginine. The precipitated

barium salts were therefore freed from barium with sulfuric acid, and the mixture of free amino acids obtained after filtration from the barium sulfate was converted into the copper salts by boiling with copper carbonate. Most of the copper salt formed was soluble in water. The insoluble part was found to consist mainly of cystine. Aspartic acid, which should appear in this fraction, could not be identified. The water-soluble fraction was freed from copper with hydrogen sulfide. The filtrate was evaporated *in vacuo*, the residue taken up in barium hydroxide solution, and alcohol added in excess. After standing several days in the ice chest, the precipitate was filtered off, dissolved in water, and sulfuric acid added until the solution was acid to litmus but still neutral to Congo red. The filtrate from the barium sulfate was evaporated to dryness *in vacuo*. The residue was taken up in water, and, by fractional precipitation with alcohol, a fraction of crystalline appearance was obtained. This crystalline material was dissolved in water and reprecipitated by carefully adding alcohol; clusters of needle-like crystals were thus obtained, which melted at 212-215° with decomposition and were found to contain barium. Analysis showed that the substance was the monobarium salt of glutamic acid which, dried at 40° *in vacuo*, still retains 1 molecule of water of crystallization which can be removed at 100° *in vacuo*.

Ba salt, m.p. 212–215°, dried at 40° *in vacuo*
4.267 mg. substance: 2.232 mg. BaSO$_4$
3.055   "   "   : 1.585 "   "
$(C_5H_8O_4N)_2Ba\cdot H_2O$.   Calculated. Ba 30.70
                              Found.   " 30.78, 30.53
Ba salt, m.p. 212–215°, dried at 100° *in vacuo*
5.035 mg. substance: 0.213 mg. H$_2$O
6.283   "   "   : 0.311 "   "
$(C_5H_8O_4N)_2Ba\cdot H_2O$.   Calculated. H$_2$O 4.02
                              Found.   " 4.23, 4.95
4.822 mg. substance: 2.621 mg. BaSO$_4$
5.972   "   "   : 3.248 "   "
$(C_5H_8O_4N)_2Ba$.   Calculated. Ba 31.98
                          Found.   " 31.98, 32.01

From the mother liquor of the above barium salt the free acid was prepared by adding sulfuric acid until the solution was faintly acid to Congo red paper. The filtrate from the barium sulfate was

evaporated *in vacuo*. The residue was then taken up in water and by careful addition of alcohol the free acid was obtained in the form of rhombic crystals which melted at 180°. Unfortunately, the amount was too small to afford recrystallizations in order to secure an absolutely pure preparation.

Free acid, m.p. 180°, dried at 40° *in vacuo*
  4.500 mg. substance: 0.013 mg. ash, 2.44 mg. H_2O, 6.61 mg. CO_2
Nitrogen (Micro-Dumas)
  3.570 mg. substance: 0.301 cc. N_2(752 mm. at 26°)
Amino nitrogen (Van Slyke)
  3.720 mg. substance: 0.640 cc. N_2(757 mm. at 22°)

Another sample of the free acid was dried at 100° *in vacuo* for 20 hours.

  4.992 mg. substance: 0.089 mg. ash, 2.70 mg. H_2O, 7.22 mg. CO_2
  $C_5H_9O_4N$.   Calculated. C 40.80, H 6.17, N 9.52
                 Found.   " 40.15, " 6.09
  Ash-free substance.   " 40.16, " 6.16
                                      N 9.52 (Dumas)
                                      " 9.66 (Van Slyke)

DISCUSSION

As can be seen from the analytical data the values obtained for the barium content of the salt correspond very well to that calculated for a barium salt of glutamic acid. The barium salt prepared from glutamic acid (Eastman Kodak Company preparation) was found to behave in a manner similar to that obtained from insulin. This salt melted at 212–215° with decomposition and showed no depression in melting point when mixed with the barium salt obtained from insulin. The barium salts of glutamic acid have been prepared by Abderhalden and Kautzsch (5). Unfortunately, these authors do not give a melting point for the barium salt. They state, however, that the salt retains water very obstinately and that it can be removed only by drying at 100° for several hours *in vacuo*. The analytical figures obtained for nitrogen of the free acid check very well with the value calculated for glutamic acid; the figures for hydrogen also agree; those for carbon are 0.6 per cent too low. This fraction which was obtained from the mother liquor of the barium salt no doubt still contained

traces of impurities such as inorganic salts and perhaps small amounts of cystine. The presence of cystine would account for the low carbon content which we obtained, even when calculated for the ash-free substance. As has already been mentioned, the amount of this fraction was too small to attempt fractional crystallization for the removal of the last traces of impurities. In spite of the low figure obtained for carbon, we feel quite justified in assuming that this fraction from insulin is identical with glutamic acid, inasmuch as the other data are in good agreement with the values calculated for this amino acid. We are inclined to believe that a part of the glutamic acid has been converted into pyrrolidonecarboxylic acid during the hydrolysis and the subsequent manipulations, which would account in some measure for the low yield.

It should also be mentioned that in previous experiments which are not reported here we tried to isolate glutamic acid from the fraction containing the bases and dicarboxylic acids, using the usual method of precipitation as the hydrochloride, saturating the solution with gaseous HCl. No precipitate, however, was obtained under these conditions.

The dibasic acid fraction gave the color reactions for β-hydroxyglutamic acid described by Dakin (4), but the specificity of these tests has recently been called into question by Harington and Randall (6).

We shall postpone further discussion of our observations until we publish our complete work on the hydrolysis of insulin in more detail.

SUMMARY

Glutamic acid has been isolated from hydrolyzed crystalline insulin, proving the presence of this amino acid in the insulin molecule.

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THE CHEMISTRY OF THE COFFEE-BEAN

I. CONCERNING THE UNSAPONIFIABLE MATTER OF THE COFFEE-BEAN OIL. PREPARATION AND PROPERTIES OF KAHWEOL*

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INTRODUCTION

Coffee has become a universal and almost indispensable beverage in the modern dietary; and according to government statistics, the importation of coffee into this country during the year 1931 amounted to 870,768 tons, which corresponds to a *per capita* consumption of 12 pounds. It is surprising in view of this extensive use of coffee that, outside of caffeine and some of its combinations, very little is known concerning many of the chemical compounds which are present in and make up the bulk of the coffee-bean. In order to secure more scientific and practical information on this subject, we have begun a systematic investigation in this laboratory of various at present unknown constituents of the coffee-bean. It is planned to include in this work not only the natural raw bean, but also the freshly roasted as well as the stale or aged roasted ground coffee. In this manner it is hoped to determine what chemical compounds are chiefly responsible for certain evident deteriorations in the quality of roasted coffee during storage or ageing.

The first part of the investigation is concerned with the nature and composition of the ether-soluble or fat constituents of the coffee-bean. This part of the work was first taken up because it seemed very probable that the staling of roasted coffee is due in part, at least, to oxidative changes of the unsaturated constitu-

ents which are in the fat.   The available published information
on the nature of the coffee fat is meager and very incomplete.
Certain constants of the coffee-bean oil have been determined and
some attempts have been made to separate the fatty acids which
are liberated when the fat is saponified.

The unsaponifiable matter which represented 10 per cent of the
fat was a dark brown semisolid.   It was very easily soluble in the
common organic solvents but it was nearly insoluble in cold pe-
troleum ether.   As will be described in the experimental part of
this communication, the material was separated into two principal
fractions; namely, (1) a small amount of a phytosterol which was
very similar to sitosterol, and (2) a large amount of a new, beauti-
fully crystalline substance which possesses unusually interesting
properties.

Phytosterols have been described previously by Juckenack and
Hilger (1) and also von Noël (2), but the products isolated by
these investigators had lower melting points and lower optical
rotation than our product.   The major part of the unsaponifiable
matter referred to above as the second principal constituent is
not a sterol, and as it has not previously been fully characterized,
we have devoted some time to a study of its composition and re-
actions.   Since this substance represents the principal constituent
of the unsaponifiable matter and a not insignificant proportion of
the coffee-bean oil, we propose to designate it by the name of
kahweol from the arabic word "qahweh" meaning coffee.

Kahweol is strongly levorotary.   It crystallizes readily from
concentrated solutions but the crystal form varies with the sol-
vent.   From acetone it separated either in the form of very large
colorless trigonal or pentagonal dense plates, while from methyl
alcohol only massive colorless quadratic plates are obtained.
From ether it crystallizes in very long, snow-white silky needles.
It is easily soluble in glacial acetic acid and on the careful addition
of water colorless prismatic needles are obtained which decompose,
however, in a short time, giving a strong purple color.

Kahweol is extremely sensitive to atmospheric oxygen, light,
heat, and especially to mineral acids, while it appears to be more
indifferent to alkalies.   In acid solutions or in the presence of
traces of acid fumes, it very quickly turns purple in color.   It is
therefore very difficult, if not impossible, to keep the pure substance

for more than a few minutes or hours, at the most, in an unaltered condition. Even when pure colorless crystals of kahweol are placed in a sealed tube filled with carbon dioxide, the color changes to a deep yellow in less than 24 hours. When the substance is kept under ordinary conditions in a glass-stoppered bottle, it very rapidly changes to a yellow color and there is a constant increase in weight most probably due to the absorption of oxygen. These changes in physical appearance profoundly affect the melting point, solubility, and the optical activity. It is evident from what has been said about the sensitivity of kahweol that determinations of its physical constants must be made within a short time after it has been isolated. The freshly prepared substance, crystallized from ether and dried in a vacuum desiccator, melts at 143–143.5° to a clear oil and in methyl alcoholic solution gives a specific optical rotation of −204.5°.

Kahweol is a highly unsaturated compound and its iodine number, determined by the Hanus method, gave results varying from 131 to 155, owing to the fact that there is a formation of an insoluble product in the determination and this formation does not take place with uniformity. With the Liebermann-Burchard reaction, it gives a bright red color which gradually changes to a permanent, deep, dull red. This coloration is so strong that even traces of kahweol, mixed with a phytosterol, will entirely mask the blue to green coloration which is given by sterols in the Liebermann-Burchard reaction.

Since kahweol is so sensitive and is so easily changed, it is practically impossible to obtain concordant results on analysis, and we have not been able to determine the composition of the pure substance with a desirable degree of accuracy. The only crystalline derivative that we have been able to prepare from kahweol is a monoacetyl compound. From the data at present available, obtained more or less indirectly, we believe that the substance possesses the formula $C_{19}H_{26}O_3$ and that it contains at least one hydroxyl group.

In order to secure a more stable form of kahweol, we tried to reduce it by catalytic hydrogenation but, unfortunately, the reduction does not proceed in a normal manner. In the reduction, about one-half of the total hydrogen is absorbed very rapidly, but after that the absorption is very slow; we have not been

able to obtain a reduction product which does not give an iodine number.

The iodine number which is obtained corresponds to approximately 1 atom of iodine, and this would indicate a substitution rather than addition on a double bond. The reduced kahweol apparently contains two hydroxyl groups, which would indicate that a carbonyl group in the original substance had been converted into a secondary alcohol. While the reduced kahweol is perfectly stable under ordinary conditions with respect to light and atmospheric oxygen, it is extremely soluble in most organic solvents, and hence it does not crystallize readily. The substance melts at 173–174°, and in methyl alcoholic solution the specific optical rotation is −67.8°. Values obtained on analysis were in agreement with the formula, $C_{19}H_{32}O_3$.

A product similar to kahweol has been isolated from coffee-bean oil by von Noël and Dannmeyer (3), and the opinion was expressed by these authors, due to a report by Schwarz and Sieke (4), that it possessed antirachitic properties, but further experiments did not substantiate this assumption. Through the kindness of Dr. A. F. Hess of New York, samples of kahweol were tested for antirachitic properties, but it was found to possess no activity whatever either before or after irradiation with ultraviolet light. Similar negative results were also obtained in The Fleischmann Laboratories by Dr. C. N. Frey. Kahweol has been tested by Dr. H. A. Mattill and H. S. Olcott for antioxygenic properties with negative results. This would indicate a possible absence of a phenolic hydroxyl group, since these investigators claim that most of the substances which they had examined and found to be antioxidants possessed a phenolic hydroxyl group.

It would seem that a substance which is so sensitive, as is kahweol, to reagents, light, heat, and oxygen, must possess some active physiological properties but, so far, all tests which have been made have given entirely negative results. Preliminary feeding experiments were also executed, through the kindness of Dr. L. B. Mendel, by Mr. W. E. Anderson, but no definite results have been obtained as yet.

Preparation of Unsaponifiable Matter

The fat was first prepared by extracting freshly roasted, finely ground coffee-beans with petroleum ether[1] in a large Soxhlet extractor. The solvent used was the Mallinckrodt product with a boiling range of 30–60°. After complete removal of the solvent by distillation in a stream of CO_2, the fat was treated with 2 volumes of acetone to precipitate any phosphatides or other acetone-insoluble material. A very slight precipitate was obtained and filtered off, the acetone completely removed by distillation, and the remaining fat placed in a vacuum desiccator. This fat gave the following constants.

Iodine No. (Hanus)......................... 96.05
Saponification No......................... 172.08
Reichert-Wollny " 0.866
Specific rotation in chloroform............. $[\alpha]_D^{25}$ = −13.24°

For the saponification, 200 gm. of fat were refluxed in an atmosphere of nitrogen with 1000 cc. of 5 per cent alcoholic potassium hydroxide for 12 hours. About 200 cc. of alcohol were then distilled and the mixture of soaps was diluted with 4 liters of distilled water. The soap solution was extracted with ether eight times, with a total of 6 liters of solvent, in order to remove the unsaponifiable matter. After drying over anhydrous sodium sulfate, the solvent was removed by distillation. The remaining residue was subjected to a second saponification by refluxing with 300 cc. of 4 per cent alcoholic potash for 1 hour. The mixture was then diluted to 2 liters with distilled water, cooled, and extracted four times with ether. The ether extract was washed with water, dried over anhydrous sodium sulfate, and distilled. A dark red semisolid was obtained which weighed 20.4 gm., which is equivalent to 10.2 per cent of the original fat. Especial care was taken to protect the unsaponifiable matter from air by using, as far as possible, an atmosphere of carbon dioxide.

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[1] All solvents used in this work were freshly distilled, and the alcohol had been purified by distillation over potassium hydroxide.

Isolation of Kahweol

Since preliminary experiments had shown that it was difficult to prepare pure kahweol in large yields, an additional quantity of fat was saponified and more unsaponifiable matter collected.

The crude unsaponifiable matter, 39.9 gm., was dissolved in 40 cc. of hot acetone and the solution was diluted with cold petroleum ether until no further precipitate was produced. The precipitate which separated as an oil was quickly converted to a crystalline mass on shaking. This solid was filtered off, washed with cold petroleum ether, and designated as Crop I. The mother liquor and washings of 200 cc. deposited a large amount of fine, silky needles which were worked up separately as Crop II.

The material which was designated as Crop I was recrystallized from acetone and petroleum ether five times, yielding slightly colored, heavy, dense plates. Kahweol is very soluble in warm acetone. When the concentrated solution is cooled, large, dense, nearly colorless, pentagonal plate-shaped crystals separate. The mother liquor from these crystals, upon concentration and cooling, deposits large, dense, trigonal plate-shaped crystals. In order to decolorize the product, it was dissolved in warm ether, and to this solution was added a previously boiled suspension of norit in ether. The suspension was then refluxed a short while, filtered, and concentrated slightly. Beautiful colorless, fine needles separated which were dried in a vacuum and weighed 5.3 gm.

From the concentrated mother liquors of Crops I and II, another lot of crystals was isolated. These were dissolved in the ethereal mother liquor of Crop I. On concentration and cooling of this solution, no crystals separated, and the solvent was distilled off in a stream of CO_2 leaving a thick, yellow, resinous mass weighing 9.0 gm. The entire mass was crystallized from hot methyl alcohol in rhombic plates by cooling the orange-colored solution in a freezing mixture. This second lot weighed 5.45 gm., and on drying *in vacuo* showed a loss in weight of 10.16 per cent. This amount corresponds to about 1 molecule of methyl alcohol of crystallization which was calculated to be 9.58 per cent on an assumed molecular weight of kahweol of 302. It was evident by its behavior during crystallization that kahweol took on some solvent of crystallization and therefore appeared in the various crystalline forms. The crystals from acetone and methanol were

photographed and drawings made from the photographs are shown in Figs. 1, 2, and 3.   In contrast to the others, the edges of the trigonal crystals were not straight but slightly irregular.
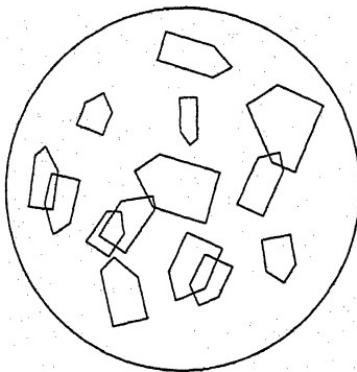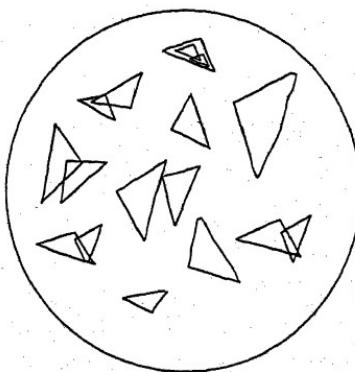

FIG. 1   FIG. 2

FIG. 1.   Kahweol from acetone.   × 27.5
FIG. 2. Kahweol from acetone mother liquor.   × 27.5
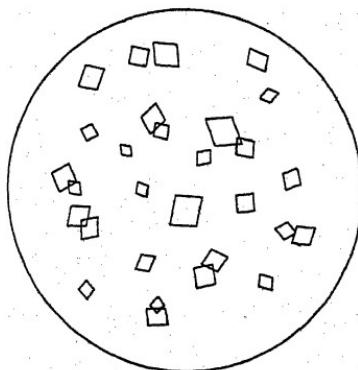
FIG. 3. Kahweol from methanol.   × 27.5

Kahweol has a satisfactory melting point, softening at 142° and melting to a clear orange-colored oil at 143–143.5° showing a slight decomposition at the melting point.   On cooling the melt does not crystallize but remains as a transparent resin.   It is very sensitive to light, heat, and air.   It turns yellow in color on exposure.   It decolorizes bromine, gives no coloration in alcoholic

solution with ferric chloride, and in the Liebermann-Burchard re-
action gives a bright red color which changes in a few minutes to
a deep dull red which remains permanent.   Qualitative tests for
nitrogen, halogen, sulfur, and ash were negative.

Attempts to obtain a molecular weight directly by Rast's (5)
method were unsuccessful due to incomplete solubility in camphor.

Rotation—0.0660 gm. of substance dissolved in methyl alcohol
and made up to 10 cc. gave in a 1 dm. tube a reading of $-1.354°$;
hence $[\alpha]_D^{21} = -204.54°$.

Analysis—4.507 mg. gave 3.22 mg. H_2O and 12.40 mg. CO_2
            Found.   H 8.00, C 75.04
            Calculated for $C_{19}H_{26}O_3$. " 8.61, " 75.50

It should be stated here that kahweol darkens on drying before
analysis at 100° in a vacuum and in an atmosphere of nitrogen.

Some of the pure white, needle-like crystals were exposed to
light and air at room temperature and almost immediately a yel-
lowish tint was observed, which developed into a decided yellow
color after several hours.   At the end of 34 days, 0.3159 gm. gained
15.4 mg. or 4.87 per cent.   On inspection of this exposed material
it was found that the discoloration occurred on the surface first
and gradually advanced towards the interior, since, upon disin-
tegration of a few small masses after this long exposure, some
colorless crystals were found.

An examination of this exposed substance also revealed a change
in melting point and optical rotation as well as solubility.   The
melting point was now indefinite, as the substance darkened at
120° and fused to minute globules which effervesced slightly at
142° but did not completely decompose with effervescence before
200°.

Rotation—0.0750 gm. of substance dissolved in methyl alcohol
and made up to 10 cc. gave in a 1 dm. tube a reading of $-1.253°$;
hence $[\alpha]_D^{21} = -167.06°$.

From the above determination it is evident that a diminution
of 37.48° in rotation had occurred.   Moreover, the exposed kah-
weol was incompletely soluble in chloroform whereas the fresh
substance dissolved completely, although on standing, a deep
green color would develop with a precipitation of a dark green
flocculent solid.

In order to ascertain some facts as to the constitution of this interesting product, attempts were made to prepare a few derivatives. Owing to its high unsaturation, the derivatives were in preference prepared from the reduced kahweol. However, an acetylation of kahweol was successfully executed.

Acetylation of Kahweol

With the method of Verley and Bölsing (6), 0.4437 gm. of kahweol was treated with 20 cc. of acetic anhydride-pyridine mixture on a steam bath for 15 minutes. After cooling and addition of 25 cc. of water, the clear yellow solution was titrated with sodium hydroxide. Fine needle-like, prismatic crystals separated out. A difference of 1.55 cc. of 0.855 N sodium hydroxide was obtained between the titration of a blank and the actual determination.

Analysis—1.55 cc. 0.855 N NaOH = 0.079515 gm. $C_2H_4O_2$
         Found. $C_2H_4O_2$ 17.92
         Calculated for $C_{19}H_{25}O_3 \cdot COCH_3$.   "   17.44

This would indicate that a monoacetyl derivative had been formed. The crystals were filtered off, thoroughly washed with water, and dried *in vacuo* overnight. The weight was 0.46 gm., and the substance melted at 132–134° to a clear orange-colored oil. The Liebermann-Burchard reaction gave the same color as the original kahweol.

The acetyl product crystallized very satisfactorily from dilute alcohol in long, slender, needle-like prisms and from 95 per cent alcohol in long, slender plates of pale salmon color. After two recrystallizations the substance sintered at 125°, softened to a turbid oil at 133–134°, and melted to a clear oil at 142–144°. A remelt occurred at 136–137°. On drying at 110° *in vacuo* the substance lost 2.84 per cent in weight. Calculated on a basis of $C_{21}H_{28}O_4 \cdot \frac{1}{2}H_2O$, the theoretical loss would be 2.55 per cent.

Analysis—3.808 mg. gave 2.65 mg. H_2O and 10.30 mg. CO_2
         Found.   H 7.79, C 73.76
         Calculated for $C_{19}H_{25}O_3 \cdot COCH_3$. " 8.20, " 73.25

The analysis indicates an empirical formula of $C_{21}H_{27}O_4$ as against the assumed formula of $C_{21}H_{28}O_4$.

For a further check on the acetyl content of this derivative, 0.1339 gm. was treated with an excess of 0.1 N alcoholic potassium hydroxide with gentle refluxing for $\frac{1}{2}$ hour. The excess alkali was titrated with standard sulfuric acid and a consumption of 4.04 cc. of 0.1 N alkali for saponification was found.

Analysis—4.04 cc. 0.1 N KOH = 0.02424 gm. $C_2H_4O_2$
Found. $C_2H_4O_2$ 18.11
Calculated for $C_{19}H_{25}O_3 \cdot COCH_3$.   "   17.44

Reduction of Kahweol

5 gm. of kahweol were dissolved in 200 cc. of alcohol, 0.2 gm. of platinum oxide which had been prepared according to the method of Voorhees and Adams (7) was added, and the suspension was shaken with hydrogen. At the start, the absorption of gas was rapid but after 500 cc. were absorbed, the action was markedly retarded. The mixture was warmed and in the course of 30 minutes an additional 400 cc. of hydrogen were taken up. The catalyst now began to separate out, so the suspension was filtered; the filtrate was shaken with fresh platinum oxide and hydrogen. Within an hour 200 cc. more of hydrogen were slowly absorbed, when the catalyst again separated. The suspension was filtered and the alcohol distilled off under diminished pressure, leaving a thick, resinous, faintly yellow residue which would not crystallize on standing overnight. A Liebermann-Burchard reaction still gave a pronounced yellowish orange color.

The resinous material was dissolved in 200 cc. of absolute alcohol, 0.1 gm. of platinum oxide was added, and the hydrogenation was repeated. There was a slow but distinct absorption of gas and, after a treatment of an hour, during which warming and strong shaking were resorted to, 150 cc. more of hydrogen were taken up, making a grand total of 1250 cc. of gas which were absorbed. Since further absorption was not evident, although the catalyst was in colloidal suspension, the hydrogenation was interrupted. The catalyst was removed by treatment with norit, the filtrate concentrated in a vacuum to a small volume and chilled in a freezing mixture, but since no crystals separated, the concentrated solution was evaporated almost to dryness with CO_2. On continued scratching, crystallization was finally effected. The mass was placed in a vacuum desiccator for 48 hours, after which time

a hard white cake weighing 6.0 gm. was obtained. Part of this increase in weight is due to alcohol of crystallization.

The solid cake was treated with 25 cc. of ether and with stirring a semicrystalline product resulted. After the substance had been filtered off, washed with ether, and dried *in vacuo*, it weighed 4.3 gm. The melting point was 170–172°; the melted substance did not crystallize on cooling.

The mother liquor yielded 1.9 gm. of a nearly colorless residue when evaporated to dryness in a current of carbon dioxide.

All attempts to crystallize the ether-insoluble reduced product from single or mixed solvents proved unsuccessful. A solution of the substance in acetone, when mixed with petroleum ether, deposited a white amorphous product which contained a few prismatic crystals. The reduced kahweol softened at 165–166° and melted to an opaque oil at 171–172° which cleared at 175°. An iodine number was obtained with the Hanus method, and no insoluble iodide was evident as was the case with kahweol. This value was 33.63 corresponding to but 1 atom of iodine.

Rotation—0.0985 gm. of dried substance dissolved in methyl alcohol and made up to 10 cc. in a 1 dm. tube gave a reading of −0.668°; hence $[\alpha]_D^{23} = -67.81°$.

Analysis—3.805 mg. gave 3.54 mg. H_2O and 10.34 mg. CO_2
       3.835 "   "   3.57 "   "   "   10.45 "   "
       Found.   H 10.41, C 74.12
                           " 10.42, " 74.31
       Calculated for $C_{19}H_{32}O_3$. " 10.47, " 74.02

The above analyses indicate an empirical formula of $C_{19}H_{32}O_3$ and molecular weight of 308.

The ether-soluble fraction of 1.9 mg. was dissolved in 15 cc. of chloroform and treated with petroleum ether until a permanent cloudiness was apparent. On scratching, crystallization began and on standing overnight, a small amount of colorless, prismatic crystals had separated on the sides and bottom of the flask. These were filtered off and the filtrate was concentrated *in vacuo* to a faintly yellow, glassy mass. There was no tendency to solidify in crystalline form. A treatment with 50 cc. of absolute ether yielded a small amount of insoluble matter which was removed by filtration and the clear filtrate was concentrated. On stand-

ing overnight, a small amount of solid had separated. The clear ether solution was decanted and concentrated, when a colorless glassy mass was obtained. This was placed in a vacuum desiccator and on evacuation, the substance suddenly solidified to a nearly colorless mass of shining, thin filaments weighing 1.8 gm. This material on rubbing presented an appearance of crystalline particles, but it could not be separated in crystalline form from any solvent.

The iodine number by the Hanus method was 121.65 but this value is only approximate, since there was a formation of an insoluble product in the reaction.

Rotation—0.1006 gm. of substance dissolved in methyl alcohol and made up to 10 cc. in a 1 dm. tube gave a reading of -0.593; hence $[\alpha]_D^{23} = -58.94°$.

Acetylation of Reduced Kahweol

For acetylation, 1.0 gm. of the ether-insoluble fraction was refluxed for 2 hours with 25 cc. of acetic anhydride and 2 gm. of fused sodium acetate. On cooling the mass was diluted with water, when an oily material, which would not crystallize, separated. It was extracted with ether; the ether extract was washed with water and dilute sodium bicarbonate and finally with water until the washings were neutral in reaction. The orange-yellow-colored extract was treated with norit, filtered, and evaporated to dryness to a thick, yellow, oily substance weighing 1.5 gm. This was placed in a vacuum desiccator overnight and formed a clear transparent resinous mass weighing 1.4 gm.

This derivative was very soluble in acetone, methyl alcohol, ethyl alcohol, chloroform, ethyl acetate, benzene, and glacial acetic acid, but would not crystallize. Petroleum ether seemed to soften the substance but did not dissolve it completely.

Since this derivative could not be crystallized, the crude substance was saponified for the purpose of determining the number of acetyl groups.

Analysis—0.2065 gm. substance required 11.65 cc. 0.1 N KOH to saponify. 11.65 cc. 0.1 N KOH = 0.0699 gm. $C_2H_4O_2$.

Found. $C_2H_4O_2$, 33.85
Calculated for $C_{13}H_{30}O_3 \cdot (COCH_3)_2$.   "   30.61

The value obtained would indicate the presence of two acetyl groups signifying the presence of two hydroxyl groups in the reduced kahweol.

Further corroboration on the presence of two hydroxyl groups was sought in the preparation of a naphthyl isocyanate derivative.

Naphthyl Isocyanate Derivative of Reduced Kahweol

In a small flask, 0.5 of reduced kahweol and 1.0 gm. of naphthyl isocyanate were mixed and heated over a low flame to nearly the boiling point. Complete solution ensued. On cooling, a transparent resinous mass remained which would not crystallize. It was warmed up again and on scratching, crystallization was effected. The mass was treated with absolute alcohol in which almost complete solution took place. A slight amount of insoluble matter was removed by filtration and water was gradually added to the clear filtrate until the solution was cloudy. On standing, an oily substance separated on the bottom of the flask. The clear supernatant liquor was decanted and the residue, after it had been dried in a vacuum desiccator, weighed 0.82 gm. This material could not be crystallized from organic solvents. It was therefore purified by dissolving in alcohol, treating with norit, filtering, and precipitating by the gradual addition of water, when an amorphous solid separated.

After drying *in vacuo*, this derivative softened at 118° and melted to a slightly turbid oil at 128°.

Analysis (Dumas Method)
0.2045 gm. substance gave 7.8 cc. N at 22° and 754 mm.
0.2037 "   "   " 7.8 " " " 22° " 752 "
              Found.   N 4.36, 4.38
              Calculated for $C_{19} H_{32}O_3 \cdot (C_{10}H_7NCO)_2$. " 4.33

Here again the analysis indicates the presence of two hydroxyl groups in the reduced kahweol.

One further determination of hydroxyl content was carried out by the Zerevitinov (8) method with the following results.

Analysis—0.1214 gm. substance gave 18.0 cc. CH_4 at 25° and 740 mm.
          0.1910 "   "   " 29.4 " " " 25° " 742 "
          Found.   OH 10.10, 10.52
          Calculated for $C_{19}H_{30}O \cdot (OH)_2$. " 11.04

The values obtained in all these determinations point towards the presence of two hydroxyl groups in the reduced kahweol leaving 1 oxygen atom unaccounted for. This second hydroxyl group most probably was formed through the reduction of a carbonyl group present in the original kahweol.

Isolation of Sitosterol

Crop II, described in an early section of the experimental part, was recrystallized from petroleum ether by chilling the warm saturated solution in a freezing mixture. This product was slowly soluble in hot methyl alcohol but dissolved completely on long heating. On cooling the methyl alcohol solution, fine colorless needles separated out. The sterol was then recrystallized twice from alcohol from which it separated in very thin plates weighing 0.68 gm.

The mother liquors were combined, concentrated, and cooled. The crystals which had separated were filtered off, washed with cold methyl alcohol, and recrystallized twice from this solvent, giving 0.44 gm. which proved to be identical with the first fraction.

The substance gave a blue color changing to a deep green in the Liebermann-Burchard reaction. It melted sharply at 138–139° to a clear oil, solidifying at 127–126° and remelting at 138°.

Rotation—0.1349 gm. of dried substance dissolved in 10 cc. of chloroform in a 1 dm. tube gave a reading of -0.480; hence $[\alpha]_D^{21}$ $= -35.58°$.

Analysis—3.855 mg. gave 4.06 mg. H_2O and 11.79 mg. CO_2
    3.943 "   " 4.18 "   "   " 12.08 "   "
Found.   H 11.79, 11.86, C 83.41, 83.55
Calculated for $C_{27}H_{45}OH$. " 11.91, C 83.93

4.042 mg., 4.135 mg. dried in nitrogen with a partial vacuum lost 0.187 mg., 0.192 mg.

        Found. H_2O 4.63, 4.64
        Calculated for $C_{27}H_{45}OH \cdot H_2O$. " 4.45

A Rast molecular weight determination was made with the following result.

Analysis—0.225 mg. substance + 2.934 mg. camphor gave $\Delta = 7.5°$
        Found.   Mol. wt. 409
        Calculated for $C_{27}H_{45}OH \cdot H_2O$. "   " 404

The properties and composition of the substance indicate that it is similar to the usual sterols found in plants.

SUMMARY

1. The unsaponifiable fraction of the fat, extracted from freshly roasted coffee, contains a highly unsaturated, strongly optically active, and sensitive product which we designate by the name kahweol, together with one or more sterols.

2. The composition of kahweol corresponds approximately to the formula $C_{19}H_{26}O_3$. The substance apparently contains one hydroxyl group and it melts at 143–143.5°; $[\alpha]_D^{21} = -204.5°$.

3. Catalytic reduction of kahweol leads to a compound having the composition $C_{19}H_{32}O_3$ in which two hydroxyl groups are present. Reduced kahweol melts at 175°; $[\alpha]_D^{23} = -67.81°$.

4. The phytosterol isolated is similar to sitosterol. It was analyzed for $C_{27}H_{45}OH \cdot H_2O$; it melted at 138–139°, and the specific optical rotation was $-35.58°$.

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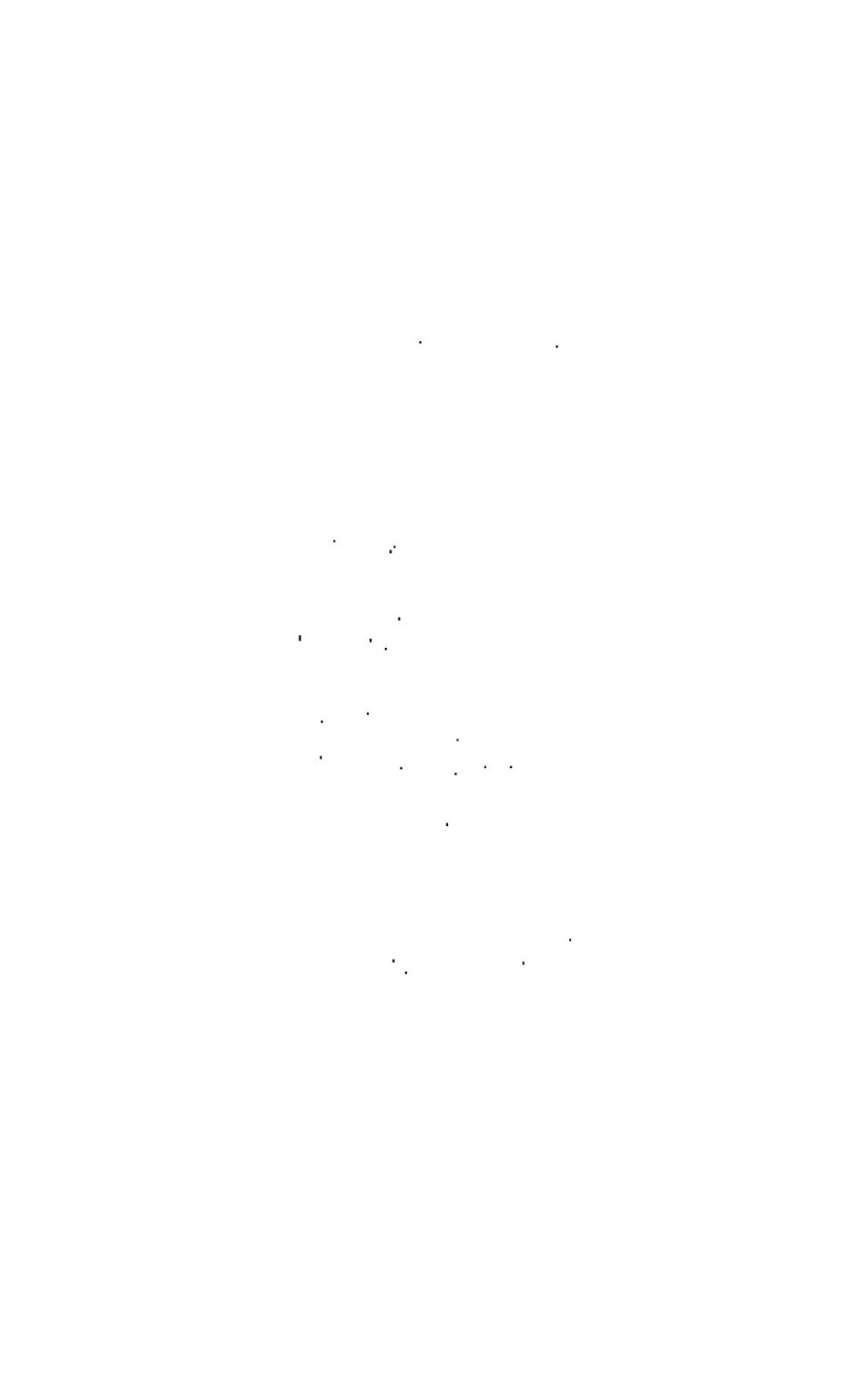
THE EFFECT OF ORAL ADMINISTRATION OF AMINO ACIDS AND INTRAPERITONEAL INJECTION OF VARIOUS ELEMENTS AND HYDROCHLORIC ACID ON REGENERATION OF HEMOGLOBIN

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The relationship of iron to anemia is a problem that has concerned investigators for a long period of time. It had its origin in connection with studies involving a comparison of the potency of inorganic *versus* organic iron, which later led to studies on the relation of solubility of iron compounds to treatment of anemia. Later still, it led to a study of ferric as compared to ferrous compounds in iron therapy and then, in the past few years, to the rôle of copper in iron metabolism. The latter phase—namely the rôle played by copper—has been the subject of investigation by a considerable number of workers, the results of whom do not all agree. Hart, Steenbock, and coworkers (1) were the first to point out that pure iron salts did not cure nutritional anemia, but that the addition of a small amount of copper salt to the iron salt resulted in hemoglobin synthesis. Keil and Nelson (2), employing extreme precautions and sensitive tests for copper, came to the same conclusion; namely, that copper plays a specific rôle in hemoglobin building and that of a large number of different elements tested it alone has the capacity to make iron available to the body in the formation of the respiratory pigment. Keil and Nelson (3), in a previous paper, partially disagreed with Hart, Steenbock, and coworkers by stating that iron could be utilized directly but the action of it was catalyzed by copper. In their most recent communication they claim that without copper hemoglobin synthesis is impossible.

It is not necessary to review all of the work on this subject, either for or against copper in hemoglobin building, since the above

citations are sufficient for the purpose at hand. Further evidence for or against the necessity of copper in the formation of hemoglobin has been presented by Lewis, Weichselbaum, and McGhee (4), Krauss (5), Elden, Sperry, Robscheit-Robbins, and Whipple (6), Drabkin and Waggoner (7), Myers and Beard (8), Mitchell and coworkers (9), Titus, Cave, and Hughes (10), Underhill, Orten, and Lewis (11), McHargue, Healy, and Hill (12), and Williamson and Ewing (13). Evvard, Nelson, and Sewell (14) conducted some investigations on the nutritional rôle of copper simultaneously with Hart, Steenbock, and coworkers and it is interesting to note that they observed that the greater part of the stored copper was confined to the liver. They stated at this early date, "It is possible that the medicinal and nutritive value of liver and its proper functioning may be somehow related to this element."

The most recent contributions to the literature on nutritional anemia and hematopoiesis have been those of Drabkin and Miller (15) and Beard and Myers (16). Drabkin and Miller showed that on the basis of daily consumption the anemia produced in rats by a diet of cow's milk can be cured by synthetic diets containing less copper than is present in milk. They observed that hemoglobin regeneration was very rapid upon a synthetic diet which was deficient in vitamins and salts and which contained only a small amount of iron. Since the quality of the protein was different in the synthetic diet as compared with milk, and since the level of protein fed per day in the synthetic diet was higher than in milk, they were led to believe that certain amino acids may play a part in stimulation of hemoglobin in this type of anemia. They found that arginine and glutamic acid proved very effective for hemoglobin regeneration in nutritional anemia, whereas certain other amino acids were far less potent or had no effect at all. Beard and Myers (16) in a series of extensive experiments have been unable to verify the specific effect of copper. They state, "The view that inorganic Fe cannot be utilized by anemic young rats for blood regeneration is not supported by the experiments recorded in this paper. . . . These findings are quite in agreement with the observations of Mitchell and Schmidt, Drabkin and Waggoner, and Keil and Nelson on the rat, and Whipple and Robscheit-Robbins, Riecker, Riecker and Winters, and Steiger on the dog. . . . " Furthermore, Myers and Beard (17) say that other

elements besides copper are effective in hemoglobin regeneration, especially so the elements Ni, Ge, Mn, As, Ti, Zn, Rb, Cr, V, Se, and Hg.

The experiments recorded in this paper were instituted in order to answer certain questions; First, do amino acids have any effect on regeneration of hemoglobin? Secondly, does iron alone, especially in high doses, have the capacity to regenerate the respiratory pigment? Thirdly, do other elements behave like copper? Last, an attempt was made to estimate the amount of Cu necessary to supplement Fe in hemoglobin synthesis. It is possible that the conflicting results with different elements may be due in part to the mode of administration of the elements. Such small quantities are fed that varying amounts may be absorbed, resulting, therefore, in varied conclusions. Hence, in this work we injected the salts intraperitoneally as well as administered them orally.

Rats were employed in all of the experiments. Anemia was produced by an exclusive milk diet. The milk was obtained from pure bred Holstein cows, and special precaution was taken to milk directly into glass receptacles, thus avoiding contamination with copper-containing metals. Keil and Nelson (2) have recently emphasized that pure $FeCl_3$ added to milk collected in tinned vessels caused regeneration, but failed to do so when the milk was directly collected in glass. All of the animals were kept in individual cages made of hardware cloth. The bottoms were composed of the same material to eliminate coprophagy. The food was furnished to the animals in mortars and all feeding utensils were washed and cleaned daily with copper-free water. Ordinary distilled water was not used for this purpose because of the danger of contamination with copper.

The amino acids used in these experiments were both purchased and prepared. It is not necessary to give the details of preparation, since the conventional methods were employed. The preparations were pure, since they conformed to the various criteria of purity generally employed for this purpose. Electrolytic sheet copper was used as the source of this element. It was dissolved in redistilled HNO_3 and then was converted into the sulfate and recrystallized from copper-free water. The $FeCl_3$ was prepared from Baker's Analyzed standard iron wire. The wire

was dissolved in concentrated redistilled HCl, and the solution was evaporated to dryness. The residue was then dissolved in 0.25 N HCl, and H_2S was bubbled through the solution in order to precipitate any copper that might be present. The solution was filtered, boiled, and a little Cu-free HNO_3 added. The solution was then made alkaline with NH_4OH and the $Fe(OH)_3$ filtered off and washed repeatedly with Cu-free water. The $Fe(OH)_3$ was dissolved in redistilled C.P. HCl. All of the solutions of iron were analyzed by the KCNS method. The various salts used for injection or feeding were tested and found Cu-free. The Fe and many of the other elements were examined by a Hilger quartz prism spectrograph and shown to be free of copper. Carbon electrodes were used in the spectrographic examination. The spectrum was developed in a 10,000 volt condensed spark between carbon electrodes. The lower electrode was hollowed out to contain the solution. Hemoglobin was determined by the Newcomer method. The animals were bled from the tail.

The following amino acids were studied: tyrosine, tryptophane, glutamic acid, aspartic acid, and arginine. Arginine and glutamic acids were fed as the hydrochloride. The animals were placed on milk collected in glass when 30 days old, and, after 1 month on this diet, were given in addition to the milk 100 mg. of the different amino acids daily plus 0.5 mg. of Fe as $FeCl_3$. The data are recorded in Chart I. The results show that none of the amino acids fed caused regeneration of hemoglobin and from that point of view the results are not in accord with the data of Drabkin and Miller (15). They confirm the work of Elvehjem, Steenbock, and Hart (18) regarding the failure of glutamic acid in hematopoiesis.

Chart I shows data on the effect of 1, 5, and 10 mg. of Fe as $FeCl_3$ daily when fed to anemic rats along with milk collected in glass. The results are not in agreement with Beard and Myers (16) or the several investigators mentioned earlier who say that hemoglobin regeneration is possible on Fe alone. Mitchell and Miller (19) recently stated that there is a slow but definite response to pure iron salts. Beard and Myers (16) say that recovery of hemoglobin in 6 weeks was obtained when the milk was supplemented with as little as 0.25 mg. of Fe daily, while 2.0 mg. of Fe appeared to give more rapid regeneration than any combination of other elements with 0.50 mg. of Fe. We failed to obtain regen-

eration on 40 times the minimum employed by these investigators. The iron employed by us was shown to be Cu-free by spectrographic examination. 10 mg. of Fe as $FeCl_3$ were the maximum we were able to add and still have the animals consume the food.
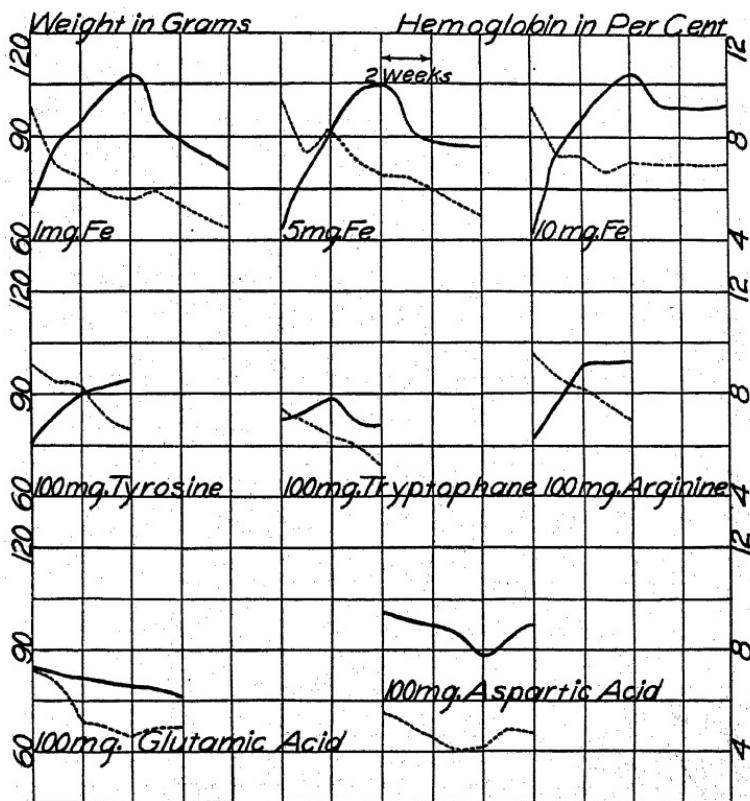

CHART I. Showing the effect of different levels of iron as ferric chloride on hemoglobin regeneration. Curves on the effect of amino acids are also shown. The animals that received amino acids were fed 0.50 mg. of iron as ferric chloride daily. The weight curves are indicated by the solid lines; hemoglobin, by the dotted lines.

Chart II shows that none of the elements when injected intraperitoneally caused regeneration of hemoglobin. Arsenic as As_2O_3 (0.01 mg. of As) caused an initial stimulation after the 1st week, which lasted for about 3 weeks. However, the initial stimulation was lost subsequently. No other element had this
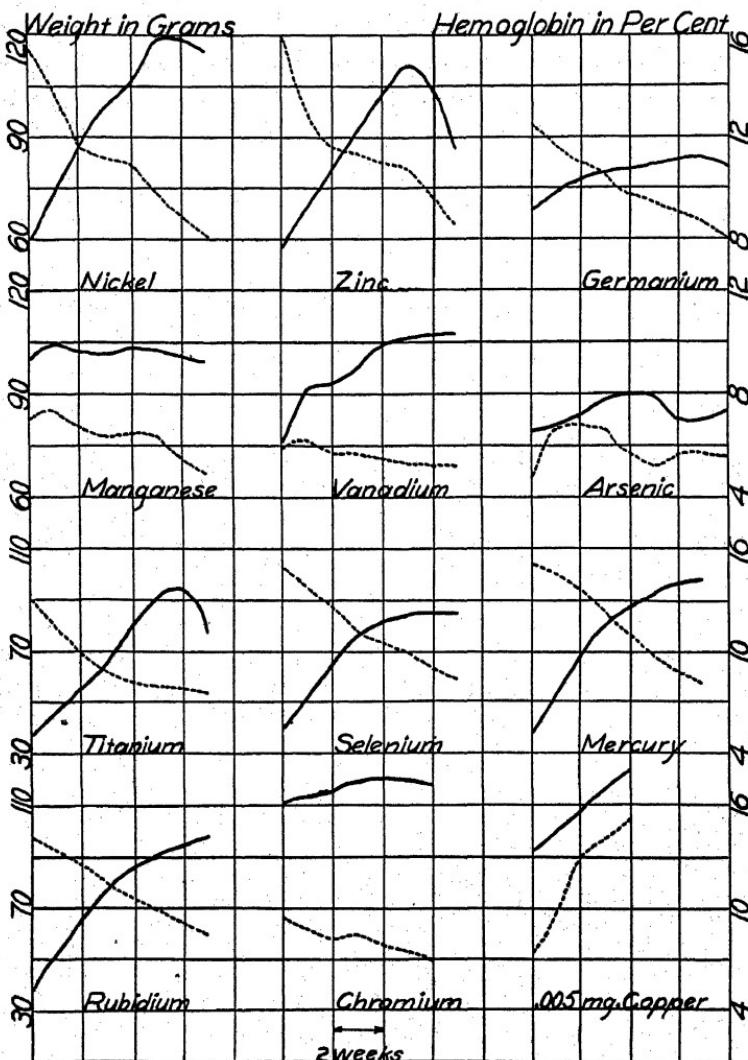
CHART II. Results of adding iron as ferric chloride to a whole milk diet, and intraperitoneal injection of various elements. Some of the animals were made anemic before injection of the salts while others were not. Nickel, zinc, manganese, rubidium, and chromium were made up as chlorides. Germanium and arsenic were used as oxides. V_2O_5 in hydrochloric acid was a source of vanadium. TiO_2 was dissolved in aqua regia. Mercuric acetate was a source of mercury. Selenic acid was a source of selenium. The weight curves are indicated by the solid lines; hemoglobin, by the dotted lines.

initial effect. It appears that germanium injections reduced the severity of the fall in hemoglobin; but this element did not prevent anemia, and, consequently, we are justified in stating that germanium cannot replace copper in the synthesis of hemoglobin.

The results obtained on oral administration of Fe as $FeCl_3$ were negative even though the intake was as high as 10 mg. of Fe per day. We were interested in the mechanism of the action of copper and in some of our work injected Fe as salts intraperitoneally. Four rats received whole milk until anemic and then were given orally 0.1 mg. of Co as the chloride together with Fe as $FeCl_3$. The Co failed to stimulate hemoglobin formation. The Co and Fe feedings were therefore discontinued. At this point the hemoglobin values were 5.5, 4.5, 4.2, and 3.0 gm. per 100 cc. of blood. 3 mg. of Fe as $FeCl_3$ in aqueous solution per rat were injected intraperitoneally every other day. 4 weeks later the hemoglobin values were 11.8, 12.0, 9.5, and 12.1 gm. per 100 cc. of blood. The ferric chloride solution was the same as that given orally at a 10 mg. level and which, when so administered, failed in hematopoiesis as shown in Chart I. Another group of three rats received whole milk until anemic, and germanium as chloride (0.10 mg. of Ge) was then added to the milk together with 0.50 mg. of Fe as $FeCl_3$. The effects of the germanium were negative. The Ge and Fe feedings were therefore discontinued. At this point the hemoglobin readings were 4.0, 3.5, and 4.5 gm. per 100 cc. of blood. 3 mg. of Fe as $FeCl_3$ in aqueous solution per rat were injected intraperitoneally every other day. The hemoglobin rose to 14.5, 9.7, and 13.0 gm. per 100 cc. of blood respectively 4 weeks later. This same $FeCl_3$ failed in hemoglobin building when fed orally at a 10 mg. level daily.

Aqueous solutions of $FeCl_3$, when injected intraperitoneally, cause lesions, necrosis, and a sloughing off of the hair and skin at the point of application. The procedure is extremely severe on the animal. The $FeCl_3$ was therefore dissolved in glycerol which greatly reduced the irritating effect of the salt so that in most of the animals no apparent external injury was manifest. Sixteen rats were employed for injection of $FeCl_3$ in glycerol. The glycerol was redistilled in glass under reduced pressure. 1 mg. of Fe as $FeCl_3$ in glycerol was injected intraperitoneally every other day to rats made anemic on whole milk. The initial readings of

hemoglobin were 7.0, 7.1, 4.5, 9.1, 6.5, 8.9, 4.8, 6.6, 6.5, 8.7, 6.0, 5.2, 6.0, 5.5, 4.3, and 5.5 gm. per 100 cc. of blood. The final hemoglobin values in the same order were 9.2, 9.3, 8.0, 10.2, 9.4, 11.5, 10.6, 6.0, 10.0, 9.5, 10.5, 7.5, 8.3, 11.4, 12.5, and 10.0 gm. per 100 cc. of blood. Nine of the animals died following the last determination of hemoglobin. The hemoglobin for the eighth animal reached a value of 9.1 gm. per 100 cc. of blood 2 weeks after the injection of the iron salt; and then the value fell to 6.0 gm. 2 weeks later, when the animal died. Likewise, Rats 12, 13, and 16 reached maxima of 9.3, 10.7, and 12.7 gm. of hemoglobin per 100 cc. of blood. The seven animals that were withdrawn from the experiment were analyzed for Cu after removal of the intestinal tract. The amount of Cu per animal as determined by the sodium diethyldithiocarbamate method varied from 0.025 to 0.028 mg. This is about one-half the Cu content of a 30 day old rat on a growth promoting ration. The animals varied in age from 14 to 24 weeks. Ferric citrate when injected intraperitoneally gives results comparable to $FeCl_3$. No irritation or necrosis results, however. Four rats made anemic on milk were given injections intraperitoneally of 1 mg. of Fe as citrate every other day. The initial hemoglobin values were, 6.5, 5.8, 6.7, and 7.7 gm. per 100 cc. of blood. The maximum hemoglobin values were 12.5, 12.0, 14.0, and 12.7 gm. per 100 cc. of blood. The citric acid was recrystallized from copper-free water and allowed to react with $Fe(OH)_3$. The same lot of ferric citrate was administered orally at a level of 3.3 mg. daily. The initial readings of hemoglobin were 5.5, 5.6, and 9.0 gm. per 100 cc. of blood. The final values shortly before death were 3.6, 4.0, and 7.8 gm. per 100 cc. of blood.

The data strongly indicate that injections of the chloride or citrate of iron cause at least a temporary rise in hemoglobin which may persist for many weeks. We are not prepared to say that the iron is utilized in hematopoiesis under these conditions to the exclusion of copper. It may mean that the differences observed on injection and oral feeding of iron salts to anemic animals may be attributed to differences in absorption of iron, and that, as the amount of Cu in the food or tissues is reduced, there is a decrease in the absorption of iron.

It is known that $Fe(OH)_3$ when administered orally can be used as a source of iron. The question arose whether it could be used

when injected intraperitoneally. Five rats were made anemic by milk feeding. They ranged in hemoglobin from 3.1 to 5.0 gm. per 100 cc. of blood and in weight from 50.0 to 87.0 gm. They were then given 0.05 mg. of Cu as $CuSO_4$ in the milk daily and were injected every other day intraperitoneally with 1 mg. of Fe as a suspension of $Fe(OH)_3$. The collodion dialysate of the $Fe(OH)_3$ suspension gave no test for Fe with KCNS. After 11 weeks the final hemoglobin values ranged from 13.2 to 16.3. There can be no question of the utilization of $Fe(OH)_3$ under these conditions. While the final values are given for 11 weeks, some of the animals reached a maximum as early as 5 weeks. When $Fe(OH)_3$ was fed orally with Cu, the maximum hemoglobin was reached in about 4 weeks. However, $Fe(OH)_3$ injected either subcutaneously or intraperitoneally did not stimulate hemoglobin formation in anemic rats fed milk without copper. The rats tolerated $Fe(OH)_3$ injections very well without any visible external injury.

Detre (20), working with rabbits, showed that hemorrhagic anemia responds to treatment with acids. When HCl, H_2SO_4, or H_3PO_4 were administered to rabbits made anemic by bleeding, there was a marked increase in the rate of regeneration as compared with the controls. The time for regeneration with acids was 16 days; whereas, it required an average of 27 days for the animals not treated with acid to reach a normal value. We were, therefore, interested to see what effect HCl would have on nutritional anemia. The acid was prepared by decomposing $MgCl_2$ with H_2SO_4 and passing the HCl so formed into copper-free water through a drying train so arranged as not only to dry the gas but also prevent the carrying over mechanically of any traces of copper. The acid was copper-free.

Four rats were made anemic with milk and had initial hemoglobin values of 6.5, 8.0, 7.4, and 8.2 gm. per 100 cc. of blood. They were then injected intraperitoneally with 1 cc. of 0.0333 N HCl every other day. After 5 weeks the hemoglobin values were 6.8, 10.2, 8.7, and 11.0 gm. per 100 cc. of blood. A second lot of five rats was treated in the same way, except 1 cc. of 0.092 N HCl was used for injection. They had initial hemoglobin values of 4.1, 4.0, 6.5, 4.3, and 7.2 gm. per 100 cc. of blood. The maximum values reached in about 3 weeks were 8.7, 7.5, 8.9, 6.7, and 9.8 gm. per 100 cc. of blood. After this time the hemoglobin fell

so that in 7 weeks the values were 8.0, 5.0, 8.6, 6.1, and 5.8 gm. per 100 cc. of blood. The acid injection was then discontinued and 0.01 mg. of Cu as $CuSO_4$ was injected every other day. After
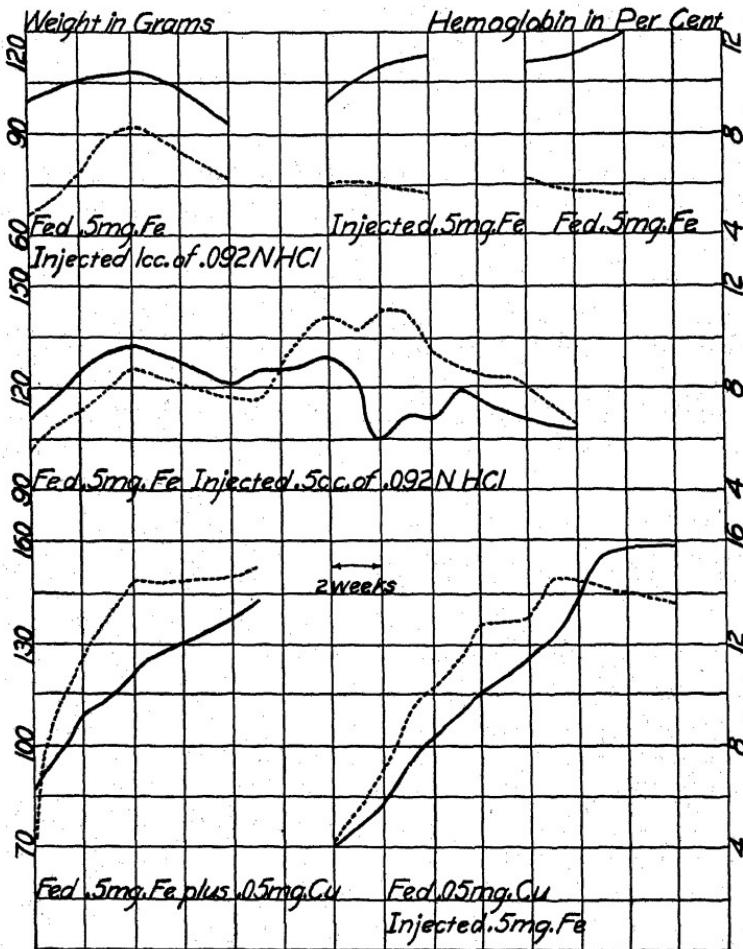

CHART III. All of the animals in the experiments here recorded received iron as ferric hydroxide. Ferric hydroxide when injected or fed is not used in hemoglobin formation except when copper or acid is administered. The effect of the acid is temporary. The weight curves are indicated by the solid lines; hemoglobin, by the dotted lines.

4 weeks the hemoglobin readings were 12.0, 15.4, 16.2, 14.5, and 15.1 gm. per 100 cc. of blood. A third group of four animals was

injected with 0.50 cc. of 0.092 N HCl every other day. The initial hemoglobin values were 6.1, 6.0, 5.7, and 5.7 gm. per 100 cc. of blood. The maximum hemoglobin levels reached in about 14 weeks were 10.3, 11.5, 12.0, and 13.5 gm. per 100 cc. of blood. The last two animals died shortly afterward. At the end of 18 weeks the first two rats had hemoglobin levels of 9.1 and 8.2 gm. per 100 cc. of blood. The first lot of rats on acid received $FeCl_3$ in the milk and the last two lots $Fe(OH)_3$ equivalent to 0.50 mg. of Fe daily. Acid apparently causes a temporary rise in hemoglobin, but sooner or later the level of hemoglobin falls. It cannot replace copper, for as little as 0.005 mg. daily of Cu injected intraperitoneally into rats which had received acid and whose hemoglobin was declining caused a marked rise in a period of 1 week. The results on $Fe(OH)_3$ and acid are shown in Chart III.

SUMMARY

Pure iron, in the form of chloride, does not cause regeneration of hemoglobin when fed to anemic rats at a level of 10 mg. daily. Tyrosine, tryptophane, glutamic acid, aspartic acid, and arginine, when fed at a level of 100 mg. daily, failed to stimulate hemoglobin regeneration in rats with nutritional anemia.

Copper is specific in its effect on hematopoiesis. Intraperitoneal injections of salts of Ni, Zn, Ge, Mn, V, As, Ti, Se, Hg, Rb, and Cr failed to stimulate hemoglobin building.

Intraperitoneal injection of HCl in anemic rats on milk and pure ferric chloride caused a temporary rise of hemoglobin.

Intraperitoneal injection of ferric chloride or ferric citrate into rats suffering from nutritional anemia caused an increase in hemoglobin.

Pure ferric hydroxide when administered intraperitoneally to anemic rats on milk and Cu stimulated hemoglobin formation.

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PHOSPHORUS DISTRIBUTION IN CHICKEN BLOOD AS AFFECTED BY THE DIET

By V. G. HELLER, K. R. HUNTER, and R. B. THOMPSON

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Phosphorus has long been recognized as one of the indispensable constituents of the diet. Many studies have been made of its metabolism because of its important functions in the growth of the cell, its rôle in the regulation of the blood neutrality, and its utilization in the formation of the bony structure of the body.

In nutrition studies with the chicken, these factors are of even greater importance because of the abnormal and artificial conditions under which the chickens are now reared and the frequent appearance of deficiency diseases thought to be produced by faulty mineral absorption.

Several studies have been made of the inorganic phosphorus and calcium content of the blood serum of the chicken or the so called calcium to phosphate ratio of the same. Russell, Massengale, and Howard (1) reported 4 to 6 mg. of inorganic phosphorus per 100 ml. of the blood serum. Hart, Halpin, and Steenbock (2) found that the number of mg. of inorganic phosphorus per 100 ml. of serum varied from 1.07 to 2.80 without cod liver oil in the diet, and from 2.50 to 5.15 with cod liver oil in the diet. Buckner, Martin, and Peter (3) pointed out that the amount and per cent of phosphorus in serum were approximately the same in weak and strong chicks. Hughes and Titus (4) reported that the number of mg. of inorganic phosphorus per 100 ml. of serum varied from 1.83 to 3.72 in chicks with leg weakness, and from 3.55 to 5.10 in chicks without leg weakness.

However, investigations of the literature fail to supply data recording the total phosphorus or, what is more interesting, the phosphorus distribution in chicken blood. This information was

desired in the Experiment Station of this institution in connection with the effect of the ration of a chicken upon its phosphorus metabolism. The excellent methods for determining the phosphorus distribution in blood, developed by Youngburg and Youngburg (5) and slightly modified by McCay (6), have made possible an investigation of this question, the results of which are recorded in the body of this article.

<div align="center">EXPERIMENTAL</div>

50 single comb white Leghorn pullets were selected from our college flock. These chickens had been hatched at the same time from eggs of similar origin. They were divided into two lots similarly housed and cared for. Lot I received a mixed grain and mash ration (Ration I) calculated to contain 0.483 per cent of phosphorus. Lot II received a similar mixture (Ration II) so prepared as to contain 1.16 per cent of phosphorus in naturally occurring forms. The composition of each ration was as follows:

	Ration I		*Ration II*
25 parts	ground oats	25 parts	bran
25 "	" barley	25	shorts
20 "	yellow corn	20	corn-meal
10 "	meat scraps	10	meat scraps
10 "	corn gluten	7	cottonseed meal
5 "	dried buttermilk	5	dried milk
3 "	calcium carbonate	3	calcium carbonate
1 part	salt	3	bone meal
1 "	cod liver oil	1 part	salt
		1 "	cod liver oil

When the chickens were about 8 months of age and had been fed these rations for 60 days, analyses were started.

The blood for analysis was secured in every case by heart puncture. About 6 ml. per bird were drawn; sufficient samples were pooled to make 25 ml. Coagulation was prevented by first coating the interior of the receiving flask with ammonium oxalate, 3 mg. per ml. of blood having been dissolved in redistilled water and evaporated in the receiver. As blood was added the mixture was vigorously stirred, iced, and sealed.

A 1 ml. sample of the whole blood was removed for total phosphorus determination and the rest was transferred to graduated

15 ml. centrifuge tubes, sealed with vaccine caps, and centrifuged at high speed for 20 minutes. The tubes were then removed, and the volume of plasma and cells was recorded. These were then separated by means of a special curved syphon and retained for analysis.

The method used in the analysis was the McCay modification of the Youngburg procedure previously referred to, being the same in every detail except in certain dilutions made necessary by the very high phosphorus content discovered in this blood. A detailed discussion having been made in the original paper, no further reference will be made to the technique in this article. In the production of the desired blue color, difficulties were often encountered by the formation of yellow or green tinges, which were later found to be produced by molybdium oxides in excess of the amount that the stannous chloride used could reduce. For this reason we are listing the dilutions found most desirable as a guide to others who may wish to apply this method to the study of chicken blood. The resulting factors used in calculations are likewise altered and are recorded for convenience.

Total Phosphorus in Whole Blood—1 ml. was diluted to 40 ml. with 0.9 per cent NaCl. A 1 ml. sample was used. Standard = 0.02 mg. of P. The colorimeter was set at 30 mm. Calculation, $\dfrac{2400}{\text{reading}}$ = mg. of P per 100 ml. of undiluted blood.

Total Phosphorus in Cells—1 ml. was diluted to 40 ml. with 0.9 per cent NaCl. A 1 ml. sample was used. Standard = 0.04 mg. of P. The colorimeter was set at 20 mm. Calculation, $\dfrac{3200}{\text{reading}}$ = mg. of P per 100 ml. of undiluted cells.

Lipoid Phosphorus in Cells—1 ml. was diluted to 20 ml. with alcohol-ether mixture. 1 ml. of filtrate was used. Standard = 0.01 mg. of P. The colorimeter was set at 30 mm. Calculation, $\dfrac{600}{\text{reading}}$ = mg. of lipoid P per 100 ml. of undiluted cells.

Inorganic Phosphorus in Cells—4 ml. were made up to 20 ml. with 10 per cent CCl$_3$COOH. A 5 ml. sample was used. Standard = 0.02 mg. of P. The colorimeter was set at 30 mm. Calculation, $\dfrac{60}{\text{reading}}$ = mg. of inorganic P per 100 ml. of undiluted cells.

Total Acid-Soluble Phosphorus in Cells—4 ml. were made up to 20 ml. with 10 per cent CCl$_3$COOH. 1 ml. of filtrate was diluted to 5 ml. with NaCl solution. A 1 ml. diluted sample was used. Standard = 0.02 mg.

of P. The colorimeter was set at 20 mm. Calculation, $\dfrac{1000}{reading}$ = mg. of acid-soluble P per 100 ml. of undiluted cells.

Total Phosphorus in Plasma—1 ml. was diluted to 40 ml. with 0.9 per cent NaCl. A 1 ml. sample was used. Standard = 0.01 mg. of P. The colorimeter was set at 30 mm. Calculation, $\dfrac{1200}{reading}$ = mg. of P per 100 ml. of undiluted plasma.

Lipoid Phosphorus in Plasma—1 ml. was diluted to 20 ml. with an alcohol-ether mixture. A 1 ml. sample was used. Standard = 0.01 mg. of P. The colorimeter was set at 30 mm. Calculation, $\dfrac{600}{reading}$ = mg. of lipoid P per 100 ml. of undiluted plasma.

Inorganic Phosphorus in Plasma—4 ml. were made up to 20 ml. with 10 per cent CCl$_3$COOH. A 3 ml. sample was used. Standard = 0.02 mg. of P. The colorimeter was set at 30 mm. Calculation, $\dfrac{100}{reading}$ = mg. of inorganic P per 100 ml. of undiluted plasma.

Total Acid-Soluble Phosphorus in Plasma—4 ml. were made up to 20 ml. with 10 per cent CCl$_3$COOH. A 1 ml. sample was used. Standard = 0.01 mg. of P. The colorimeter was set at 30 mm. Calculation, $\dfrac{150}{reading}$ = mg. of acid-soluble P per 100 ml. of undiluted plasma.

The inorganic content of cells and plasma was determined at once. The alcohol-ether extracts were volatilized over an electric lamp drier. The remaining samples were treated with the sulfuric acid heated in an open drier overnight to prevent bumping. The tubes were then heated at the low heat of an electric hot-plate in an inclined position until charred, and cooled. 4 drops of perchloric acid were added to each of the tubes, after which they were digested at full heat for 30 minutes. The solutions were then invariably found to be uniformly clear.

The determinations were repeated at bi-monthly periods during the year, precautions being taken not to bleed the same hen more than once a month. No particular discomfort seemed to be experienced by the flock, their health and egg production being normal throughout the year. Over twenty complete determinations of each of the lots have been made over a period of 6 months. As no noticeable variations other than the usual experimental fluctuations were recorded, the average amounts of phosphorus in mg.

per 100 ml. of each of the various fractions were calculated. Table I records the average mg. of phosphorus per 100 ml. of the original blood sample, calculated by multiplying the mg. per 100 ml. of sample by the cell to plasma ratio. (The blood of the chickens in Lot I consisted of 37.7 per cent cells, while Lot II contained 36.23 per cent cells.)

TABLE I

Phosphorus Distribution in Chicken Blood (Averages)

Phosphorus readings are given per 100 ml. of blood.

	Lot I	Lot II
	mg.	*mg.*
Total P in whole blood.	108.5	114.6
Cell, total P	85.2	85.2
" lipoid P	8.36	8.49
" inorganic P.	0.687	0.743
" acid-soluble P	29.72	29.60
Plasma, total P	24.56	28.71
" lipoid P	15.78	20.49
" inorganic P	1.96	2.89
" acid-soluble P	3.18	3.94

CONCLUSIONS

An examination of the data of Table I and a comparison of these results with those obtained in making a phosphorus distribution of blood of mammals demonstrate the very high phosphorus content of chicken blood, which in this case is 3 to 4 times as great as that of mammals.

An inspection of the inorganic phosphorus of the plasma, which is approximately that which has been used by other investigators in their serum studies, shows that it is only an insignificant fraction of the whole blood phosphorus. The very high phosphorus content of the cells compared to that of the plasma is quite in contrast to that of the calcium contents of cells and plasma, in which case the reverse condition is always found.

It becomes evident that increasing the phosphorus content of the ration results in a corresponding increase in the phosphorus content of the blood.

The senior author wishes to acknowledge the helpful suggestions of Dr. C. M. McCay of Cornell University in the original application of these methods.

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AVITAMINOSIS

XI. THE SPECIFIC EFFECT OF VITAMIN B ON GROWTH AS EVIDENCED BY THE USE OF VITAMIN B CONCENTRATES*

By BARNETT SURE

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In previous studies on the specific effect of the vitamin B complex and of vitamin B on growth (1) and lactation (2, 3) the source of the vitamin B or the vitamin B complex was Northwestern dehydrated yeast, introduced to the extent of 10 per cent in the ration. During the past year our work was extended by replacing the large amounts of yeast in the diet by dextrin and supplying vitamin B daily to each animal, separately from the ration, as concentrated extracts from rice polishings.

The biological technique employed has already been described (1), which, briefly stated, consists in restricting, daily, rats of the same sex, weighing 40 to 60 gm., to the same volume of water and amount of food consumed by their litter mates receiving the diet deficient only in vitamin B or the vitamin B complex, but allowing the animals on the restricted diets, in addition, a daily allowance of a vitamin B concentrate. Since the plane of nutrition was controlled, the difference in growth must be attributed to the *specific* influence of the vitamin B or its factors, depending on whether vitamin G was furnished in the ration.

We have recently found it difficult to destroy all traces of vitamin B in the Northwestern yeast by such drastic treatment as autoclaving the yeast at its natural pH for as long as 6 hours, at 15 pounds pressure, and it was found necessary to autoclave it for 8 hours at 25 pounds pressure as suggested by Salmon (4). However, we now find that the yeast supplied by the Standard

Brands Incorporated,[1] autoclaved at 6 hours and 15 pounds pressure, fed at a 15 per cent plane of intake, will furnish an abundance of vitamin G and only traces of undestroyed vitamin B.

In all experiments on uncomplicated vitamin B, 10 per cent Northwestern yeast, autoclaved for 8 hours at 25 pounds pressure, furnished vitamin G in the ration.

The results of this investigation are shown graphically in Charts I to VI inclusive.

The concentrates were all prepared from rice polishings. Concentrate 12 is a charcoal concentrate, prepared by extraction with
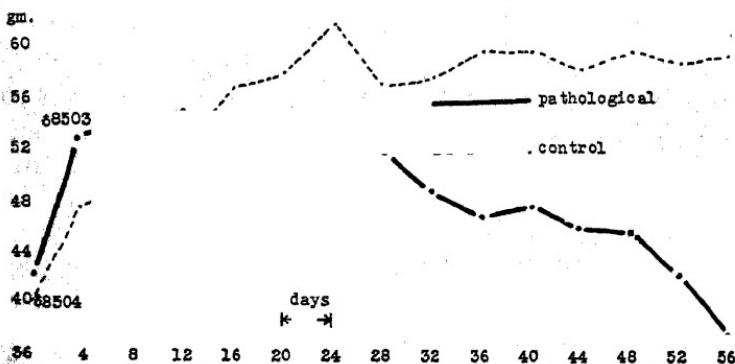
CHART I. The specific effect of the vitamin B complex on growth. Rat 8503♂ received a diet deficient only in the vitamin B complex. Rat 8504♂ was restricted daily to the same volume of water and amount of food of the diet consumed by litter mate Rat 8503, but in addition received daily, during the first 14 days, 2.5 mg. of Concentrate 12, and during the subsequent period, 1 mg. daily of Concentrate 13.

dilute alcohol, removal of inert ingredients with glacial acetic acid and anhydrous acetone, adsorption on activated charcoal, and elution with dilute hydrochloric acid, followed by elution with dilute alcohol containing 1 per cent hydrochloric acid. Concentrate 13 was prepared from Concentrate 12 by precipitation with phosphotungstic acid in a 5 per cent sulfuric acid solution. The phosphotungstates were dissolved in aqueous acetone and decomposed with amyl alcohol and ether in a hydrochloric acid solution. The small amount of material insoluble in aqueous

[1] Makers and distributors of Fleischmann's yeast, New York.
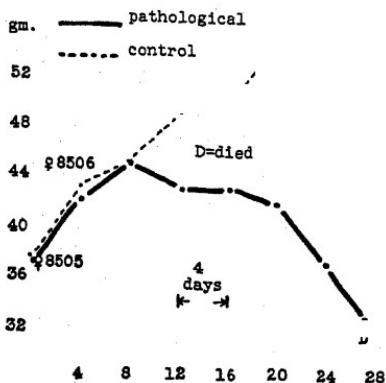
CHART II. The specific effect of the vitamin B complex on growth. Rat 8505 ♀ received a diet deficient only in the vitamin B complex. Rat 8506 ♀ was restricted daily to the same volume of water and amount of food of the diet consumed by litter mate Rat 8505, but in addition received during the first 14 days 5 mg. of Concentrate 12, and during the subsequent period, 1 mg. daily of Concentrate 13.
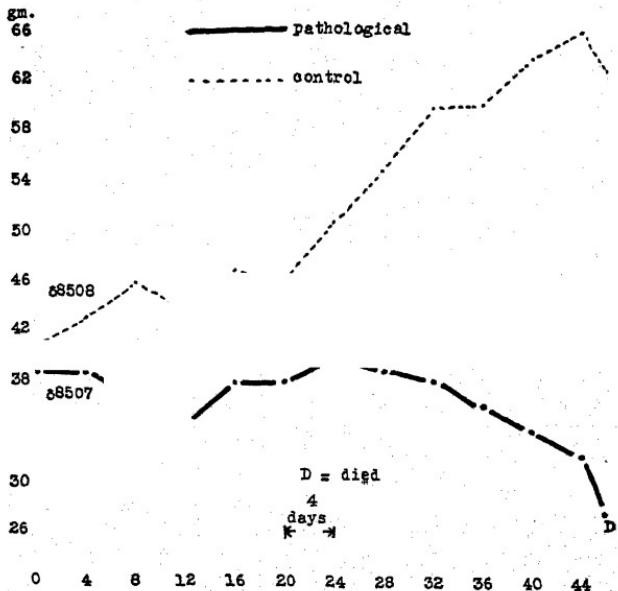

CHART III. The specific effect of the vitamin B complex on growth. Rat 8507♂ received a diet deficient only in the vitamin B complex. Rat 8508♂ was restricted daily to the same volume of water and amount of food of the diet consumed by litter mate Rat 8507♂, but in addition received daily 1 mg. of Concentrate 13.

acetone was removed by filtration before being decomposed with amyl alcohol and ether.

The biological assay of Concentrate 12 was conducted on several hundred animals by the Sherman and Spohn method (5) (as recently modified to represent a growth of 3 gm. per animal per week
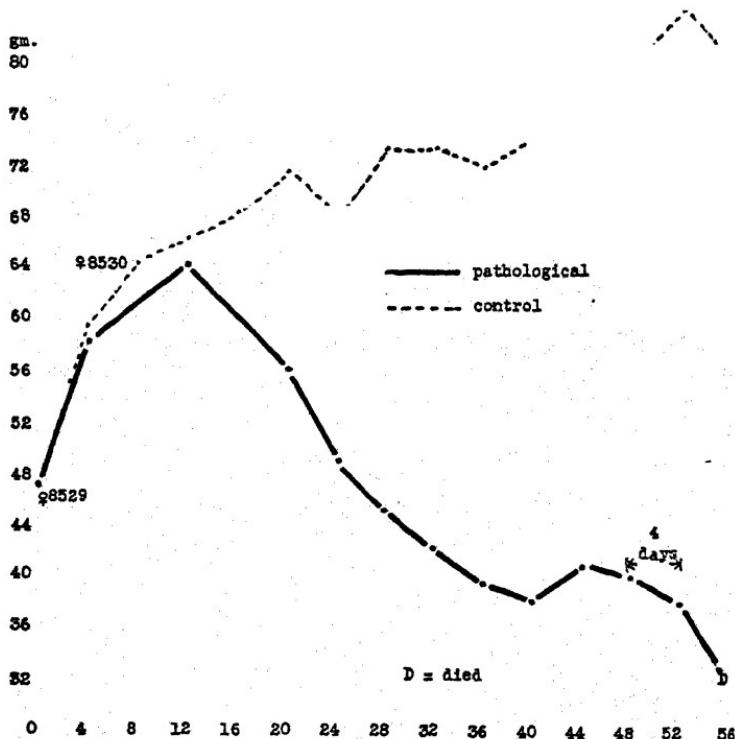

CHART IV. The specific effect of vitamin B (B₁) on growth. Rat 8529 ♀ received a diet deficient only in vitamin B (B₁). Rat 8530 ♀ was restricted daily to the same volume of water and amount of food of the diet consumed by litter mate Rat 8529 ♀, but in addition received daily, beginning on the 14th day, 1 mg. of Concentrate 13.

for a period of 8 weeks) and the average results show that, calculated on the solid basis, it is 20,000 times as potent as cow's milk in vitamin B, the most recent figures of MacLeod, Brodie, and MacLoon (6) being used for the vitamin B content of milk as a basis of comparison. No biological assay was conducted on Concentrate 13, due to lack of sufficient material, but growth experi-

ments indicated a concentration of about 5-fold, compared with that of Concentrate 12.

In order to conserve space, only a few typical illustrations are herewith submitted.

An analysis of all the charted data is unnecessary, since they are self-explanatory and require little discussion. Reference will, therefore, be made to only two experiments in which the smallest daily vitamin dosage was employed.
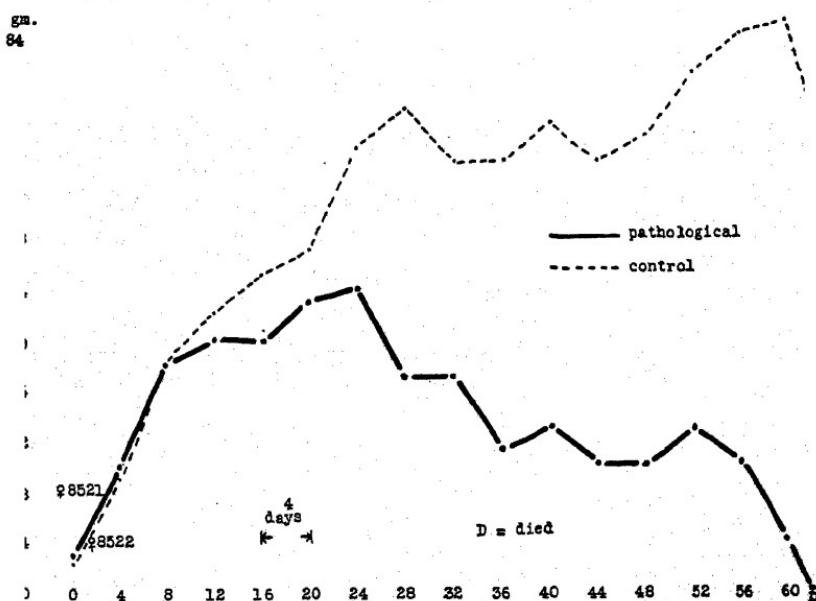

CHART V. The specific effect of vitamin B (B₁) on growth. Rat 8521 ♀ received a diet deficient only in vitamin B (B₁). Rat 8522 ♀ was restricted to the same daily volume of water and amount of food of the diet consumed by litter mate Rat 8521 ♀, but in addition received daily, beginning on the 19th day, 1 mg. of Concentrate 13.

As shown on Chart III, Rat 8507 ♂, which was on a diet deficient only in the vitamin B complex, died on the 47th day, while Rat 8508 ♂, which was on the same diet and the same plane of nutrition as its litter mate, Rat 8507 ♂, but which in addition received 1 mg. daily of Concentrate 13, was in perfect state of health on the day its litter mate died, having gained 22 gm. in weight.

From Chart IV it is clear that, while on a diet deficient only in vitamin B, Rat 8529 ♀ died on the 55th day, having lost 13 gm. in weight, Rat 8530 ♀ on the same daily volume of water intake and the same amount of the diet consumed by litter mate, Rat 8529 ♀, because it (Rat 8530 ♀) received in addition 1 mg. daily of Concentrate 13, survived and was in excellent state of health
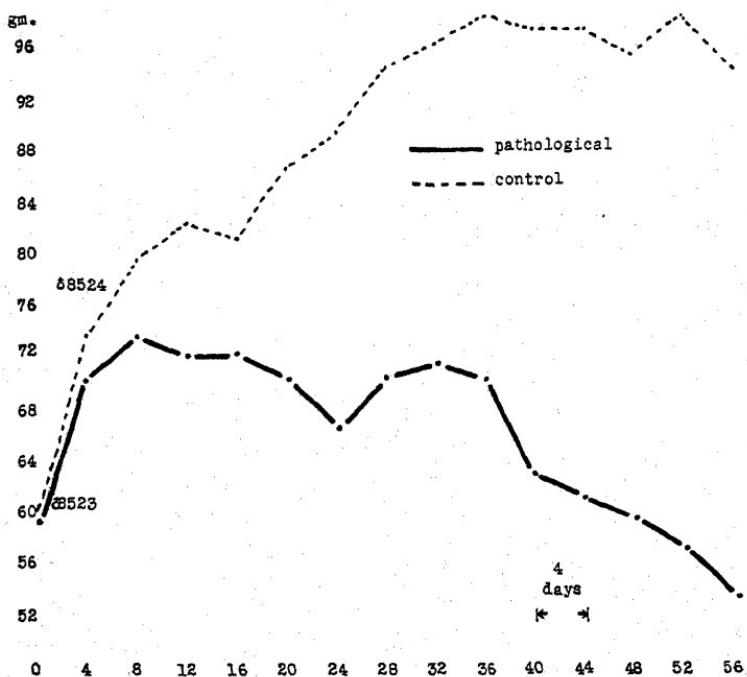

CHART VI. The specific effect of vitamin B (B₁) on growth. Rat 8523♂ received a ration deficient only in vitamin B (B₁). Rat 8524♂ was restricted to the same daily volume of water and amount of food of the diet consumed by litter mate Rat 8523♂, but in addition received daily from the beginning of the experiment 1 mg. of vitamin B Concentrate 13.

when its litter mate died, having gained 35 gm. or over 75 per cent of its weight during the period of the experiment.

The decline in growth, during the last few days of the experiment, of the animals that received the vitamin B concentrate, is due to the fact that litter mates, during the last stages of the avitaminosis, had reached a condition of either marked inanition or

fasting, and since they were restricted to the same plane of nutrition as the pathological rats, it is remarkable how well they tolerated the partial or complete fasting without showing any symptoms of undernutrition.

It has been generally assumed, although no experimental evidence has been presented, that vitamin B produces growth only indirectly by stimulating the appetite. It is now apparent from the results submitted in this paper and in recent communications (1–3) that this vitamin also exerts a specific influence on growth, unrelated to the plane of nutrition, in the same sense as certain amino acids and certain mineral elements exert their influence on growth. When, however, the animal is given free access to food and water, then greater increments of growth follow, due to the influence of vitamin B on stimulation of the appetite.

SUMMARY

1. Vitamin B produces growth in two ways: (a) It stimulates the appetite, so that there is an increase in food consumption. (b) It produces a *specific* influence on growth, unrelated to the plane of nutrition, and in this respect, is essential for growth in the sense as certain amino acids and certain mineral elements are indispensable for growth.

2. As little as 1 to 2 mg. of vitamin concentrates, as a source of the vitamin B complex, produced a *specific* influence on growth.

3. As little as 0.5^2 to 1 mg. daily of vitamin B concentrates as a source of vitamin B (B_1) produced a *specific* influence on growth.

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[2] Because of lack of space, detailed data on the 0.5 mg. daily dosage have not been included in this paper.

THE DETERMINATION OF AMMONIA IN BLOOD AND OTHER BIOLOGICAL FLUIDS

By OTTO FOLIN

(*From the Biochemical Laboratory of Harvard Medical School, Boston*)

INTRODUCTION

In 1912 Folin and Denis (1) published a series of methods for the analysis of blood and urine, all based on the principle of Nesslerization, and among those methods was one for the determination of ammonia in blood. By the help of this method Folin and Denis proved the then important point that the relatively high ammonia content of portal blood is mostly putrefactive ammonia absorbed from the gut. After having further found by a general survey that nothing else of physiological or clinical significance could be elucidated by the method we lost interest in it, and we failed to grasp the fact that the blood ammonia values found by the new method might be too small to account for the very large quantities of ammonium salts occurring in urine. Several years later, in 1921, Nash and Benedict (2) seemingly proved by substantially the same method that the ammonia found in urine must be liberated in the kidneys. Bliss (3), at my suggestion, repeated the essential parts of the work of Nash and Benedict and confirmed their finding that the blood of the renal arteries contains less ammonia than the blood of the renal veins. Later, Bliss continued the work from a new point of view, and he has endeavored to prove that there is probably ammonia formation in all tissues, but that this ammonia, originally used to neutralize acids formed in the tissues, is carried in the blood in the form of amide nitrogen contained in the blood protein of the corpuscles from which it is liberated as ammonia in the kidneys. This paper has no bearing on the controversy over the validity of Bliss' findings and interpretations.

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Parnas (4) and collaborators have determined the ammonia in blood and tissues by a somewhat different method. They remove the ammonia from blood by steam distillation in a partial vacuum and they claim that by this means they obtain more dependable values.

The ammonia in blood, according to all these different investigators, though always very small, is subject to considerable variations. Most of the figures for the ammonia in systemic blood run below 0.1 mg. per 100 cc. and many figures, particularly those reported by Nash and Benedict and by Parnas, are as low as from 0.03 to 0.05 mg. Even lower figures, 0.01 and 0.02 mg. per cent, have been obtained by Mann and Bollman, by the Nash-Benedict procedure.

For several years I have been aware that one somewhat serious error was made in the elaboration of the aeration-Nesslerization system of analysis which was published in 1912, and the effect of this error should be particularly noticeable in the determination of the minute amounts of ammonia present in blood. In connection with the methods of 1912, we abandoned the use of the special ammonia absorption tube which I had devised many years earlier for the aeration-titration method (5). We failed to realize that the quantitative extraction of very small amounts of ammonia by the help of a rapid air current is more difficult, instead of less difficult, than the recovery of large amounts (several mg.). In our check work we probably very seldom used less than 1 mg. of ammonia and probably never used less than several tenths of a mg. We thus failed to find the important fact that the losses of ammonia, due to incomplete absorption in the receiver, may be almost as large in absolute quantity when working with 0.05 mg. as when working with 0.5 mg. A technically important error was therefore made when we replaced the effective special ammonia absorption tube by one consisting only of a straight glass tube with a perforated bulb at the lower end.

Isolation of Ammonia by Air Currents

One of the first steps in the present research was to adapt the aeration-Nesslerization method for the determination of small amounts of ammonia to the use of the special absorption tube. The original tube, even when made entirely of glass, is much too
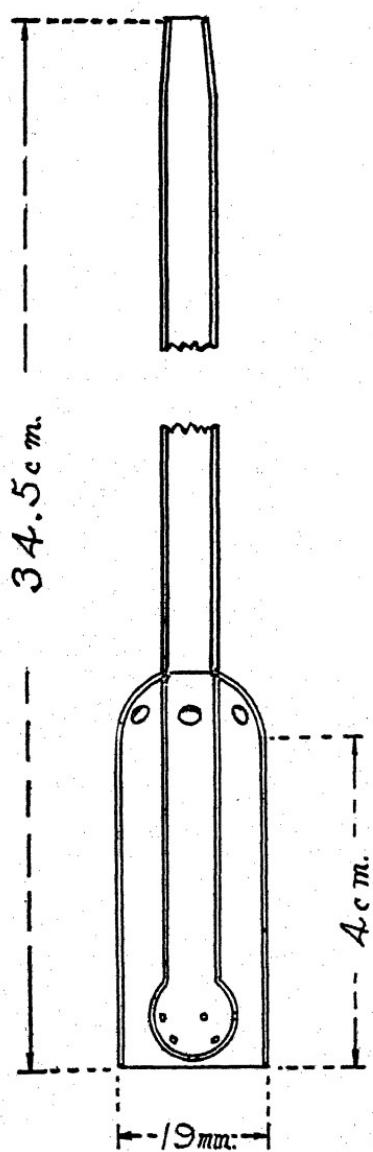
FIG. 1. Ammonia absorption tube

large for use with test-tubes, but a series of smaller ones was made, and no difficulty was encountered in getting them small enough for use with test-tubes having an internal diameter of 20

to 22 mm. These small ammonia absorption tubes, made of Pyrex glass, are just as effective as the original larger ones. They are made for us by the Macalaster Bicknell Company, Cambridge, Massachusetts. Fig. 1 is a diagram of the tube.

Having once made and perpetuated an error with regard to the absorption of ammonia from a rapid air current, I have now endeavored to determine just how much reliance can be placed on the new smaller absorption tube. For this purpose I made use of an excellent gas meter, borrowed from the Nutrition Laboratory, Carnegie Institution of Washington. This precaution seemed particularly necessary because our air supply (outside air) is delivered in practically unlimited quantities under a pressure of about 20 pounds to the sq. inch. The essential points found in the course of these tests can be briefly stated.

1. From 1 mg. to 10 mg. of ammonia nitrogen were quantitatively recovered from the fastest air current which it was practical to use—12 liters per minute.

"Quantitatively recovered" in this case does not mean absolutely quantitative. Slight losses must have occurred, but these were so small that they escaped detection in the determinations. The 1 mg. quantities (or less) were determined by Nesslerization, the large quantities by titration.

2. With 0.05 mg. nearly one-third fails to be absorbed by 10 cc. of water plus 1 cc. of 0.1 N acid when the speed of the air current is 14 to 15 liters per minute.

3. With an air current of 8.5 liters per minute, a slight loss, about 5 per cent, may be encountered when one is working with 0.05 mg. of ammonia nitrogen.

4. 7 to 8 liters per minute represent, therefore, the maximum speed of air current which will permit the complete recovery of 0.05 mg. of ammonia nitrogen by the use of the new ammonia absorption tube.

For actual work it is best to use only a speed of from 6 to 7 liters per minute. A speed of only 4 liters per minute is somewhat too slow to be effective for the removal of ammonia from 10 cc. of blood according to the process described in this paper.

The ammonia absorption tube is sufficiently small so that it might be used with the micro-Kjeldahl digestion tubes as receivers. But though there is distinctly less spattering with the new absorp-

tion tube than with a simple plain bulb tube, the maximum speed permissible with the digestion tube as receiver is only about 5 liters per minute. It is therefore better to use special tubes as receivers; mine are about 260 mm. long and have an outside diameter of about 25 mm. They are graduated at 25 cc. and at 50 cc.

After having thus found an adequate remedy for the treacherous uncertainty in the aeration method for isolating very small amounts of ammonia, it seemed worth while to reexamine the determination of ammonia in blood, a determination which represents the most exacting application of the aeration-Nesslerization principle. There were one or two added reasons for making such a critical study, one of which was that I hoped to be able to introduce one important modification applicable to all kinds of biological fluids.

The Foaming Problem

No generally adequate procedure has been found for eliminating foaming in connection with the distillation or aeration of colloidal solutions and this difficulty has always tended to limit analytical procedures. My recently described antifoaming reagent, excellent as it is for micro distillations, has proved inadequate when used in connection with the rapid aeration of blood. Other well known antifoaming reagents are unsatisfactory, because they are themselves quickly removed by the air current and in part collect in the receiver. The foaming difficulty is particularly great with such prolonged and repeated aerations of blood as may be necessary for critical studies. The viscosity of blood might well serve to retard the complete removal of the last traces of ammonia and this interference may be further increased by the mechanical obstruction offered by the millions of corpuscles which are present. Finally, some of the ammonia might be within the blood cells, and one does not know how rapidly this part of the blood ammonia will come out under the experimental conditions employed. Nor does laking of the blood help much, since it must involve either a considerable dilution or a greatly increased viscosity. It would therefore seem important, at least for check work, that one should be able to continue the aeration process until one is obtaining only the ammonia which is due to decomposition. It has been practically impossible to do this because of the foaming.

These considerations have led me to try a new, or rather an old but almost forgotten, principle applicable to the determination of ammonia. In the Schlösing method for the determination of ammonia in urine, the latter was left with a suitable alkali in a desiccator and above the liquid was placed a small dish containing standard acid for the absorption of the gradually escaping ammonia. Shaffer (6) showed nearly 30 years ago that under really suitable conditions this primitive method could yield fairly good results in the course of 2 or 3 days. It is rather remarkable that no one ever proposed to improve that old method by passing a slow air current through the desiccator and into a receiver containing the acid.

There is only a slight resemblance of the process described here to the Schlösing method, but like the latter, it depends to a certain extent on a large surface area for the escape of the ammonia. Instead of violently agitating the blood in a test-tube, by means of a rapid air current, I now sweep it out by letting the air current play over the blood in an Erlenmeyer flask. This sweeping process is not more efficient than the agitating process; it is possibly a trifle less efficient; but the important point is gained that even with the most rapid air current scarcely a single air bubble and not a single drop of spattering are encountered. For 10 cc. of blood, I use 300 cc. Erlenmeyer flasks, the bottoms of which have a diameter of about 8 cm.

In order to secure the maximum effect of such an air current, the delivery tube is drawn out to a point 1 to 2 cm. long, the internal diameter of which is about 1.5 mm. This point never touches the blood, the tip is about 2 to 3 mm. above the surface, but the force of the current is so strong that it pushes the blood away from the center, thus exposing the bottom of the flask in a circle, the diameter of which depends on the speed of the current. Under the given conditions the size of this circle furnishes an adequate practical index of the speed of the air current.

A 4 liter current will produce a clear circle about 0.7 cm. in diameter, while a 7 liter current will produce a circle about 1.5 cm. in diameter, and 15 liters per minute will give a circle with a diameter of 4 cm.

By means of this modification it should be possible to isolate the ammonia from any kind of colloidal solution. Even soap solution,

probably the most effective known foam producer, can be aerated by this process. The method has one limitation. It is absolutely essential, for rapid aerations, that the depth of the fluid shall not be much greater than that obtained from 10 to 15 cc. in a 300 cc. Erlenmeyer flask.

Which Alkali Should Be Used for Liberating the Ammonia Present in Blood?

For the mere removal of ammonia from a solution by air currents one can use an alkali as weak as a mixture of 1 part of carbonate to 3 parts of bicarbonate. But to remove every trace of the ammonia in the course of an hour under such conditions one must apply air currents of greater speed than is permissible, if every trace of the ammonia is to be collected in a receiver. After a great many experiments with carbonate-bicarbonate mixtures I have come back to carbonates as the most suitable.

In the course of this work I have tried the alkaline borate solutions so highly recommended by Parnas. We have long used solutions of ordinary borax for the isolation of small amounts of ammonia by distillation in blood urea determinations and it is possible that alkaline borates can be used at the intermediate temperatures prevailing in Parnas' steam distillations. But for use in connection with the removal of ammonia by aeration the borate solution which I tried is less effective even than a mixture of 1 part of carbonate and 3 parts of bicarbonate. My borate solution contained 9.4 gm. of boric acid and 105 cc. of N NaOH in 250 cc. This is a supersaturated solution, but the excess borate is easily brought back into solution by a very little warming.

For many purposes, potassium borates should prove more serviceable than sodium borates, because of their much greater solubility, but for the liberation of ammonia, even highly concentrated potassium borate solutions are inferior to the carbonates. The superiority of the carbonates may be due in part to the fact that CO_2 as well as the NH_3 is easily driven off by air currents.

The results obtained in two typical series of comparative experiments are given in Tables I and II. In these experiments the air current was adjusted at the beginning to the stated speeds per minute, and was not interrupted until all the determinations had been finished. The ammonia, from the standard ammonium sul-

fate solution, was contained in 10 cc. of solution, in a 300 cc. Erlenmeyer flask.

Nesslerization

The somewhat elaborate provisions described by Folin and Denis for the Nesslerization of the ammonia obtained from 10 cc.

TABLE I

Illustrating Recovery of 0.05 Mg. of Ammonia N by Air Current of 7 Liters per Minute, and Relative Efficiency of Different Alkalies

Aeration time	Alkali added	Per cent recovered
min.		
20	0.2 gm. Li_2CO_3	101
10	0.2 " "	84
15	0.2 " "	93
15	2 cc. carbonate-oxalate solution	85
15	2 " borate solution	71
20	2 " " "	80

TABLE II

Illustrating Rate at Which 0.05 Mg. Ammonia N Is Isolated by Air Currents of 4 Liters and 6 Liters per Minute When 2 Cc. of Carbonate-Oxalate Solution Are Added to 10 Cc. of Ammonia Solution

Time	Speed of air current	
	4 liters	6 liters
min.	*per cent*	*per cent*
10	55	74
15	70	85
20	90*	98†

* Residue in Erlenmeyer flask was about 0.005 mg. of NH_3-N.
† Residue in Erlenmeyer flask was about 0.001 mg. of NH_3-N.

of blood represent an early stage in the development of quantitative Nesslerizations, and they have become quite superfluous especially since the Nessler's reagent used at that time has long since been replaced by the reagent of Folin and Wu. It has been pointed out repeatedly that there is no need for getting turbid or smoky solutions instead of crystal-clear ones when Nesslerizing, but beginners are still publishing modifications designed to facili-

tate the production of clear solutions (7). In these circumstances, it seems best to call attention once more to the remarkable power of gum ghatti solutions to prevent the formation of turbidities (8). 2 or 3 drops of a 2 per cent gum ghatti extract are all that I ever use, although I have recommended as much as 1 cc. These gum ghatti solutions have proved very useful in the check work on the quantitative isolation of minute amounts of ammonia. For example, in working with a possibly ineffective ammonia absorption tube, incomplete recovery of the ammonia might be due either to inadequate aeration or to incomplete absorption. By Nesslerizing the aerated solution one can tell at once whether any ammonia is left and if so, how much. But if the carbonate-oxalate mixture is used as an alkali, Nesslerization of the mother liquor will give a turbidity almost immediately except in the presence of a little gum ghatti, whereas with only 2 drops of the latter present the Nesslerized mother liquor will remain clear for hours. Our gum ghatti solutions are preserved with about 0.1 per cent of benzoic acid which is introduced in the form of a 20 per cent alcoholic solution (5 cc. per liter).

When Nesslerizing the minute quantities of ammonia which may be obtained from blood, and especially when Nesslerizing the almost imperceptible traces which may be obtained from second and third aerations, there is more or less danger of getting turbidities unless the gum ghatti solution is used. In the presence of the protective colloid the solutions always remain perfectly clear, even when left overnight.

The Determination

There is really little to be said about the form of blood ammonia determination as I now use it which has not already been mentioned.

Transfer 10 cc. of blood to a clean, dry Erlenmeyer flask. Add 2 cc. of potassium oxalate-carbonate solution (10 per cent potassium carbonate (K_2CO_3) plus 15 per cent potassium oxalate). Insert a clean 2-hole rubber stopper carrying two glass tubes. The tube reaching to within about 1 cm. of the bottom of the flask is drawn out to a point having an internal diameter of about 1.5 mm. It connects with a large bottle containing 5 volumes per cent of sulfuric acid, where the air is washed free from am-

monia by passing through an ammonia absorption tube. The other glass tube connects with the ammonia absorption tube in the receiver. The receiver, a test-tube 260 mm. by 25 mm., is graduated at 25 cc. and contains 1 cc. of 0.1 N acid, together with water enough to reach the upper openings in the absorption tube. Start the air current and regulate it to a speed of about 6 liters per minute (5.5 to 6.5 liters). The bottom of the Erlenmeyer flask is exposed by a circle having a diameter of 1 to 1.2 cm. Keep the air current running at this speed for 40 to 45 minutes.

To each of two test-tubes, graduated at 25 cc., add 0.01 mg. and 0.007 mg. respectively of ammonia nitrogen, and to each add 1 cc. of 0.1 N acid, together with water enough to give a volume of about 22 cc.

Rinse the ammonia absorption tube with about 9 cc. of water, contained in a 10 cc. volumetric pipette, on the outside and on the inside through the opening in the top and through the large openings in the outside jacket of the tube.   Add 2 drops of gum ghatti solution to each of the three tubes and then add 2 cc. of Folin-Wu Nessler's reagent to each of the three tubes.   Make up to volume, mix, let stand for about 15 minutes (or longer if desired), and make the color comparison in the usual manner if the 0.01 mg. standard can be used.   For values less than 0.07 mg. per cent, straight test-tube comparisons are fully as reliable as those made by help of the colorimeter, but they will require additional standards.

In check work involving more than one aeration period the quantities of ammonia obtained after the first aeration will be too small for the ordinary colorimetric comparisons against the 0.01 mg. standard. For these comparisons it is best to use test-tubes of equal size graduated at 25 cc. Pour the Nesslerized unknown into one test-tube and compare with the color obtained from 0.002 to 0.006 mg. of ammonia nitrogen in other similar tubes. This comparison should, of course, be made by looking down through the full length of the test-tubes. With a little experience, one can easily determine in this way the ammonia content of the unknown to the last 0.001 mg. corresponding to 10 times as much per 100 cc. of blood.   It is particularly important to let these solutions stand for at least 15 minutes before making the final comparison, and here it is also important to use gum ghatti solution to prevent the formation of any turbidity.

In connection with the determination of 0.01 mg. of ammonia nitrogen, which is the amount, in round figures, to be expected from 10 cc. of blood, it is of course essential that no ammonia be introduced with the chemicals. The most important source of error in this respect is the potassium oxalate, used partly to prevent clotting of the blood, and even more important in the preparation of the alkaline oxalate-carbonate solution. The preparation of potassium oxalate absolutely free from ammonia is a simple matter, if one has a continuously available supply of compressed air.

Transfer 100 gm. of neutral potassium oxlate and 500 cc. of distilled water to a 1500 cc. Florence flask, add 20 cc. of 10 per cent solution of potassium hydroxide, and pass an air current through it, at 4 to 6 liters per minute, for 24 to 36 hours; *i.e.*, until 5 cc. of the solution fail to give a trace of color with Nessler's reagent. Transfer the solution to a 2 liter beaker, add with stirring 1000 to 1200 cc. of alcohol, cool for a couple of hours, and filter with suction on a Buchner funnel. Wash three or four times with alcohol and two or three times with ether. Dry in a protected place. Yield, 85 to 90 gm.

The potassium oxalate so prepared has one especial merit besides that of being completely free from ammonia. It is very light and fluffy instead of compact and heavy like ordinary powdered oxalate and therefore goes into solution more quickly. This is helpful in the prevention of coagulation of the blood, at least when the minimum safe quantity is used (20 mg. for each 10 cc. of blood). It is neutral in reaction as the added KOH is washed away with the alcohol.

For the preparation of the carbonate-oxalate solution it is not worth while to use the ammonia-free potassium oxalate, because the potassium carbonate is also likely to yield ammonia. A fairly good reagent is obtained by boiling down to 80 cc. a solution of 15 gm. of potassium oxalate and 10 gm. of potassium carbonate in 150 cc. of water and diluting it to 100 cc. Solutions so prepared give invariably a slowly developing color with Nessler's reagent, due to some impurity seemingly present in all brands of potassium carbonate, and it is neither removed by boiling nor destroyed by gentle ignition.

A reagent which gives absolutely no color with Nessler's reagent,

even after several hours, can be made by the following almost equally simple process.

Dissolve 10 gm. of anhydrous potassium carbonate and 15 gm. of potassium oxalate in about 100 cc. of water in a 300 cc. Erlenmeyer flask. Add to this solution 0.1 to 0.2 cc. of bromine and shake until all of the bromine globules have dissolved. If any precipitate is formed, as may happen with some samples of bromine, filter on a quantitative filter paper into another 300 cc. flask. Dilute the straw-yellow filtrate (or solution) to about 150 cc., and boil down to a volume of 80 or 90 cc. Cool and dilute to 100 cc.

By this treatment, the ammonia in both the oxalate and the carbonate, as well as the unknown disturbing impurity of the carbonate, is completely destroyed and the surplus hypobromite is destroyed during the boiling.

Test—Transfer 2 drops of 2 per cent gum ghatti solution and about 10 cc. of the carbonate-oxalate solution to a clean testtube. Add 2 cc. of Nessler's reagent. No trace of color should develop in the course of an hour.

It will be noted that, in the revised method described in this paper, the aeration is continued for 40 to 45 minutes, although 0.05 mg. in 10 cc. of water can be quantitatively recovered in 20 minutes. While I have made similar recovery experiments with ammonia added to blood, I think that the significance of such recoveries is not quite conclusive. Added ammonia seems to be recovered more easily than a part of the ammonia already present, hence it has seemed a little better to find the correct aeration period by studying the rate at which ammonia continues to escape from the blood during more prolonged aeration periods. These experiments have shown that the escape of ammonia never comes to a sharp and definite end. The ammonia due to decomposition in fresh blood is small; it usually amounts to no more than 0.01 or 0.02 mg. per 100 cc. of blood, in 30 minutes, and it may be even less than that during the first 30 minute period. The second 30 minute period, on the other hand, will usually yield more than 0.02 mg. per cent.

It is on the basis of such observations that I have finally come to adopt 40 to 45 minutes as the most dependable single aeration period.

Table III gives a few figures showing the amounts of blood ammonia obtained in successive 30 minute periods.

A large number of blood ammonia determinations have been made during the past season by the method described in the preceding pages. The results obtained differ from those recorded in the literature in only one respect. The conspicuously low values, less than 0.05 mg. per cent, have disappeared. In the light of this experience, I must frankly express my skepticism as to the accuracy and validity of many of the blood ammonia values reported in the literature, and I hope that Parnas will not

TABLE III

Showing That Last Trace of Preformed Blood Ammonia Is Not Always Obtained in First 30 Minute Aeration Period and Illustrating Occurrence of Some Ammoniacal Decomposition

Kind of blood	NH_3-N, mg. per 100 cc.			
	Period 1	Period 2	Period 3	Period 4
Dog 1..........................	0.075	0.04	0.02	0.02
" 2, renal vein................	0.26	0.05	0.02	0.025
" 3...........................	0.09	0.04	0.015	0.015
Human..........................	0.116	0.055	0.03	0.025
"	0.084	0.05	0.025	0.02
"	0.092	0.045	0.025	0.02
"	0.11	0.05	0.025	0.025
"	0.075	0.04	0.02	0.02
"	0.06	0.015	0.015	
"	0.095	0.035	0.015	
"	0.08	0.025	0.01+	

take it amiss, if I specifically mention one peculiar set of values of his as illustrating the point. I refer to the paper in which he proves that the ammonia content of the blood in the arm is demonstrably and unmistakably increased by exercising the hand (9). Here all of the fourteen ammonia nitrogen values obtained with the subjects at rest fell between 0.02 and 0.04 mg. per cent.

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THE BASAL METABOLIC RATES OF VEGETARIANS

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The work of Benedict and Roth (1), published in 1913, indicated that there is little if any consistent difference between the basal metabolic rates of vegetarians and non-vegetarians. It was only after observing several cases of surprisingly low basal metabolic rates in vegetarian girls of normal health and intelligence that the writers of the present paper concluded that the *length* of the period of vegetarianism might be a factor in determining the basal metabolic rate.

The specific dynamic action of proteins is a generally recognized effect. The immediate influences of sudden changes in protein ration upon basal metabolic rates have, however, been conflictingly reported. Perhaps the most spectacular case in recent literature is that of Wishart (2) who observed, in his own case, an increase of 50 per cent in his basal metabolic rate after changing from a daily protein ration of 30 gm. to one of 150 gm. He confessed, however, that the application of his ingenious formula "to the prediction of the basal metabolism of a number of individuals . . . met with little success." Wang and Hawks, in their excellent review of the whole subject (3), after citing the somewhat conflicting evidence of Kleitman (4), Wishart (2, 5), Deuel *et al.* (6), and others, "failed to find any noticeable difference in the basal heat production in a careful study of the influence of high and low protein diets on the metabolic changes in six normal adult women." The junior author of this paper (L. O. H), working on himself, reached a similar conclusion.

The object of the work recorded in the present paper was to determine whether long periods of vegetarianism could be shown to have any consistent effect upon average basal metabolic rates. The difficulties usually encountered in obtaining the services of a

sufficient number of human subjects to form the basis for a valid statistical analysis were in this case augmented by the rarity of genuine lifetime or long time vegetarians. The scope and extent of the project are indicated by the tables and the list of acknowledgments at the end of the paper.

No subjects were used who, after medical examination, showed any physical or pathological abnormalities. As a convenient means of comparison, the Du Bois normal standards were employed in calculating results. A further check on the work done in Boulder was made by determining the basal metabolic rates of twenty-six non-vegetarian students. The McKesson Metabolor and the Benedict-Roth apparatus were used for most of the tests. In Europe all of the determinations and calculations were made by the local technicians, and the results merely checked over by the senior author. It should be stated that none of the subjects was a vegetarian in the strictest sense, as they all used milk freely, and occasionally eggs. Most of the lifetime vegetarians, however, had never tasted flesh, fish, or fowl in any form, and the long time vegetarians, save when otherwise indicated, had been equally strict in their dietary habits since becoming vegetarians. 66 of the 70 subjects used in the whole investigation were staff members in various vegetarian institutions, and their dietary, while not entirely uniform, was always of the low protein type. In most cases the subjects slept in a special room, after early, light evening meals, and the determinations were made in the morning before they left their beds. The environmental conditions of diet, occupation, etc., were more nearly uniform than is usually possible in experimental work on human subjects. The wide distribution of the subjects among various nationalities should also be noted.

Results

Table I gives the results obtained for twenty lifetime vegetarians, varying in age from 18 to 65 years, six males and fourteen females, distributed among five nationalities. The maximum basal metabolic rate observed in this group was 8 per cent below the Du Bois normal and the average rate 11 per cent below. The average rate of the five American lifetime vegetarians was 13.8 per cent below the Du Bois normal, or 10.8 per cent below the averages of twenty-six non-vegetarian subjects taken on the same

machine and under the same conditions. While basal metabolic rates may vary, normally, to the extent of ±10 per cent, this consistent difference, in so large a group, is undoubtedly significant.

Of almost equal interest are the data of Table II, showing the results obtained with part time vegetarians; *i.e.*, subjects who had been vegetarians for various periods of time. It appears that

TABLE I

Basal Metabolic Rates of Twenty Normal Lifetime Vegetarians

Subject	Age	Sex	Native of	No. of tests	Average rate
	yrs.				
M. W..........	24	F.	United States	7	−10
L. D..........	21	"	" "	9	−11
A. M..........	20	"	" "	5	−16
V. B..........	19	"	" "	3	−15
B. E. M.......	23	"	" "	2	−17
M. C..........	18	"	England	2	−8
E. H..........	21	M.	"	2	−9
R. W..........	22	F.	"	2	−11
N. N..........	19	"	"	2	−9
S. P..........	23	"	Switzerland	1	−12
W. M..........	32	M.	"	6	−8
M. C..........	40	"	"	2	−9
M. M..........	30	F.	"	2	−11
V. M..........	40	M.	Germany	1	−11
P. A. D	65	"	Canada	3	−10
S. M..........	32	F.	Switzerland	2	−10
W. A..........	24	M.	"	1	−9
M. W..........	43	F.	"	2	−9
M. M..........	22	"	"	1	−8
D. F..........	51	"	"	2	−16
Average..					−11

periods of vegetarian habit up to 5 years or so are not likely to have any effect on the average basal metabolic rate. Longer periods, however, seem to be effective, and the average rate of vegetarians of 10 years standing or more was approximately the same as that of lifetime vegetarians. No vegetarian of 8 years standing or more showed a rate above −5 per cent. The data of Table II are graphically presented in Chart I. No statistical

Basal Metabolic Rates of Forty-Three Part Time Vegetarians

Subject	Age	Sex	Native of	No. of tests	Yrs. of vegetarianism	Average rate
	yrs.					
S. O.	38	F.	Switzerland	1	5	+4
O. G.	26	"	"	1	1	+9
S. M.	22	"	"	2	4	+1
S. Q.	34	"	"	1	4	−3
S. G.	25	"	"	2	2	−10
S. R.	23	"	"	2	3	−4
S. C.	28	"	"	1	10	−7
S. L.	21	"	"	1	3½	+4
A. S.	28	M.	United States	4	14	−14
A. C.	23	"	" "	2	14	−6
M. F.	24	F.	" "	2	½	+4
G. S.	33	"	" "	1	⅓	−6
F. M.	19	M.	" "	2	1	−2
E. W.	22	F.	" "	2	1½	+4
B. M.	34	"	" "	1	2½	+2
W. C.	31	"	" "	1	1½	−5
A. D.	28	M.	" "	3	9	−6
J. A.	26	F.	" "	3	9	−10
M. R.	41	"	" "	2	10	−13
F. A.	32	M.	England	2	1	−7
K. K.	25	F.	"	2	8	−11
A. A.	42	M.	Germany	3	10½	−11
C. B.	51	"	"	2	8½	−8
M. H.	29	F.	"	1	5	−7
C. S.	23	"	"	1	5	+1
C. B.	28	"	"	1	3½	−2
S. L.	34	"	"	2	6	±0
E. S.	35	"	"	1	6½	+2
S. H.	38	"	"	2	3½	−6
L. C.	48	M.	"	3	14½	−8
L. H.	28	"	"	2	4	+8
P. C.	41	"	France	2	6	−6
H. S.	38	"	"	1	6½	−5
C. B.	19	F.	"	2	4	−8
C. O.	41	M.	Denmark	5	6½	−11
N. J.	22	F.	"	2	13	−7
A. A.	25	"	"	3	12½	−8
H. W.	22	"	"	2	12	−15
V. M.	24	"	"	3	7	−13
S. A.	24	"	"	1	7½	−2
M. A.	24	"	"	1	8	−5
P. V.	27	"	"	2	11½	−7
R. A.	28	"	"	5	11	−13
Average of twenty vegetarians.....................				1–5		−1

accuracy is claimed for Chart I, but it seems significant that the graph representing the mean of the basal metabolic rates is nearly horizontal during the first 5 years, drops rapidly during the next 5 years, and levels out during the last 5 years.

The following tabulation, giving the results for seven Americans, habitual (lifetime or long time) vegetarians who ate meat not more than once a month and usually less frequently, seems to indicate that a lapse now and then from the strict vegetarian
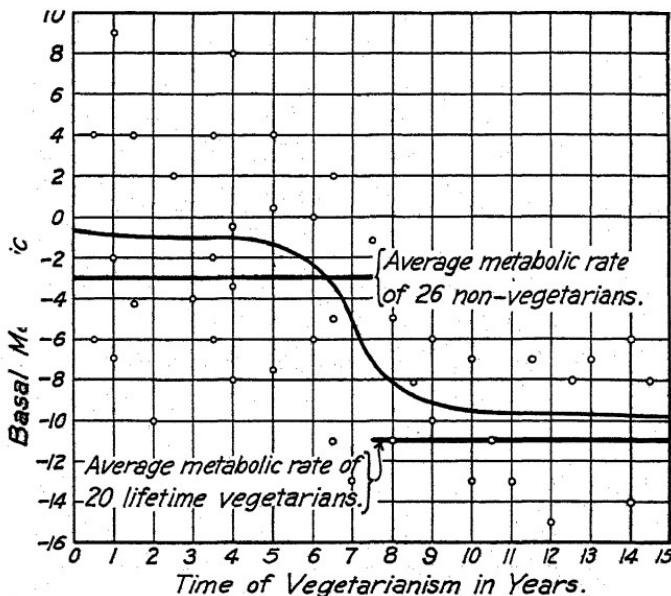
CHART I. Basal metabolic rates of forty-three vegetarians

habit does not influence the general effect of vegetarianism upon the average basal metabolic rate.

Subject	Rate
M. R.	−16
E. W.	−15
P. W.	−7
D. D.	−13
L. P.	−11
S. K.	−14
E. B.	−16
General average	−13

The average basal metabolic rate of twenty-six non-vegetarian American subjects taken under equivalent conditions on the same apparatus was −3.

TABLE III

Basal Metabolic Rates of Twenty-Six Non-Vegetarian American Women. Control Group

Subject	Age	Basal rate	Subject	Age	Basal rate
	yrs.			yrs.	
M. A.	21	−9	D. O.	20	−8
R. B.	20	+4	D. B.	25	+3
P. B.	19	−3	B. W.	19	−11
M. C.	20	+10	T. S.	23	+8
P. G.	24	+2	D. K.	27	−2
J. I.	21	−4	G. W.	24	−10
M. J.	19	−5	M. G.	21	−7
R. K.	19	+3	M. A.	24	−11
E. M.	21	−9	G. M.	27	−2
M. M.	21	−2	H. P.	20	−12
M. R.	21	−4	A. A.	22	+2
N. T.	21	−9	M. S.	23	+3
O. O.	21	0			
E. S.	20	+1	Average		−3

TABLE IV

Basal Metabolic Rates of Six Swiss Non-Vegetarians. Control Group

Subject	Age	Sex	Basal rate
	yrs.		
D. M.	37	M.	+10
F. O.	18	F.	−4
L. C.	31	"	+5
A. O.	41	M.	−2
S. P.	24	F.	+7
R. R.	21	"	−6
Average			+2

Tables III and IV give the essential data of two control groups—one in Colorado and one in Switzerland. Only one determination could be made in each case, which may account for the wider range of values found. This range, of course, overlaps that of the vege-

tarian group, but the sizes of the two groups seem sufficient to give statistical validity to the difference between the two averages, notwithstanding the wide individual variations. These, in fact, do not exceed those commonly observed in basal metabolic work on normal subjects.

DISCUSSION

The significance of these results is not at all clear. The long period of time required to produce the observed effect eliminates the possible influence of differences in digestibility, absorption, etc. It seems clear that some rather profound effect upon actual cell metabolism is indicated. Perhaps it may be tentatively suggested that the cells of the organism, confronted over a long period of time with a low supply of amino acids, gradually adjust their metabolic processes in such a way as to increase the mechanical efficiency of the body as a heat engine and thus enable the organism to carry on its normal functions with less loss of the total potential energy of the food in the form of heat.

It should be stated that the sublingual temperatures of forty-two of the subjects—all of the cases in which this factor was observed— were always between normal limits, 36.7–37.2°.

While no physical or psychological tests were made, it can be confidently stated that the subjects used in this investigation were at least up to normal averages in physical health, strength, and endurance as well as in mental intelligence and alertness. They included physicians, technicians, nurses, and office employees and many of them were athletic.

SUMMARY

The average basal metabolic rate of twenty lifetime vegetarians was found to be 11 per cent below the Du Bois normals.

Study of a large group of long time vegetarians indicated that a period of from 6 to 8 years of vegetarianism is usually required to produce this effect.

Occasional lapses from strict vegetarianism on the part of habitual vegetarians do not affect the average rate.

ties, the services of their technicians in making determinations, and their cordial cooperation in permitting their staff members to serve as subjects in this investigation: The Boulder-Colorado Sanitarium-Hospital; The Cancer Hospital, Free, London, England; The Stanboroughs, Watford, Herts., England; La Lignière, Gland, Canton Vaud, Switzerland.

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THE RENAL THRESHOLD OF BILIRUBIN

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Determination of the renal thresholds of the different blood constituents in man—the concentrations of these substances in the blood at which they appear in the urine—is difficult for a number of reasons. Experiences with glucose may be cited as an example of the many possible physiological and technical variables. It is, perhaps, the difficulty of controlling these variables which has made it doubtful whether the renal threshold concept is applicable to any blood constituent; and bilirubin affords no exception. This blood pigment presents additional difficulties in that it does not lend itself very well to ingestion, because of the destructive action of the secretions and bacteria of the gastro-intestinal tract. Nor does bilirubin readily lend itself to intravenous injection, because of its solubility properties and the difficulty of simulating in the injected fluid the physical state in which bilirubin normally circulates in the blood stream.

The problem is further complicated by the present status of our knowledge of the van den Bergh reaction—the only practical method at present available for the detection of bilirubin when present in very small quantities. Van den Bergh did not regard the method as strictly quantitative, but considered it sufficiently so to afford a fairly accurate index of progress of individuals suffering from jaundice. Fischer and Barrenscheen (1), unable to obtain complete coupling of the diazo reagent with simple solutions of bilirubin, concluded that the procedure is unreliable for quantitative work. More recently, however, Hunter (2) has shown that, under suitable conditions, coupling of diazobenzene-p-sulfonic acid with bilirubin is complete; that with simple solutions there is

strict proportionality between the amount of bilirubin and intensity of color. With sera, provided the conditions outlined are adhered to strictly, this reagent supplies a method of highest accuracy; added bilirubin could be recovered quantitatively. Suitable dilution of sera containing large amounts of pigment was stressed. Proper dilution not only tends to minimize loss of pigment by adsorption on to the precipitated blood proteins, but also to keep the amount within the limits of concentration at which bilirubin will couple quantitatively with the diazo reagent. Sera should be diluted so that they will contain no more than about 3.0 mg. of bilirubin per 100 cc.

In the absence of other substances in the reacting mixture which might also couple with the diazo reagent with development of a color similar to that produced by bilurubin, specificity of reaction is of secondary moment. An important consideration here, therefore, is that bloods of individuals suffering from jaundice—subjects necessary for the present investigation—contain many pigments other than bilirubin. This is suggested by the variety of skin discolorations; the colors, as is well known, vary from a light yellow to a deep olive-green. Since this range of colors is largely due to products closely allied chemically to bilirubin, it is important to determine the degree of specificity of the diazo reagent.

Ehrlich believed that the diazo reagent used in this test was specific for bilirubin; other pigments (oxidation products of bilirubin), biliverdin, bilifuscin, biliprasin, etc., gave no color reaction. Though this seems to be the consensus of opinion, a number of recent observers appear to have found otherwise. Davies and Dodds (3) claim that biliverdin gives a color reaction, but slowly. Roberts (4) also noted a similar reaction; and Dastre and Floresco (5) and Küster (6) found that coupling occurred with other oxidation products. Hunter (7) believes that Ehrlich's reagent is specific for bilirubin and suggests that the differences mentioned above were due to contamination of the oxidation products used with bilirubin; biliverdin, believed to be pure, gave a negative reaction. Newman (8) confirmed the slow reaction noted by Davies and Dodds with biliverdin, and Griffiths and Kaye (9) observed a somewhat similar color reaction but state definitely that "it cannot be biliverdin."

In view of the conflicting results of direct experiments, it appears

justifiable to approach the problem *indirectly*. It may here be observed that lack of specificity of the diazo reagent, if it exists at all, does not necessarily exclude its use. Experiences with studies of the renal threshold for glucose may be cited as an example. There is, as yet, no specific method for the quantitative estimation of blood sugar; all estimations, more or less, include other reducing substances. As by far the greater part of the reducing substances of blood is glucose, the non-specific nature of the different blood sugar methods does not alter their practical value. These observations are applicable to the van den Bergh reagent. If it could be shown, assuming the oxidation products of bilirubin give a color reaction with the diazo reagent, that the color develops more slowly or is of such intensity that the greater part of the color given by a sample of blood is due to bilirubin, the method could still be made use of. This phase of the subject was, therefore, investigated.

The underlying principle of a variety of tests, as the Gmelin, Rosenbach, and Huppert-Cole, for qualitative detection of bile pigments is oxidation with formation of a series of color derivatives. The last named test may, as one of its authors points out (10), be made an extremely sensitive procedure. An important fact made use of in this test is the solubility of the oxidation products of bilirubin in acid-alcohol. Essentially the same principles were employed by Hooper and Whipple (11) in their quantitative estimations of the bile pigment in bile, and our many experiences confirm the reliability of this procedure. The limits of error are, at least, within those necessary for the present purposes. A comparative study of the results obtained with this test and the van den Bergh reaction with a solution containing a rich mixture of all bile pigments (bile) should, therefore, afford an indication of the relative degree of coupling of the diazo reagent with bile pigments other than bilirubin.

The technique for the estimation of *total* bile pigments is as follows:

Two 100 cc. volumetric flasks are half filled with the oxidizing reagent.[1] To one flask is added, drop by drop, 1 cc. of bile.

[1] This reagent is a 95 per cent solution of ethyl alcohol, containing 4.0 cc. of concentrated nitric acid and 20 cc. of concentrated hydrochloric acid per liter.

The mixture is then diluted to the mark with oxidizing reagent. 1 cc. of the standard[2] is then added to the other flask and this is also diluted to the mark with the reagent. Both flasks are then well shaken and allowed to stand for about 18 hours. It will be observed that, in time, the precipitated proteins of the bile settle to the bottom of the flask and leave a clear supernatant fluid. The colors change at first to an intense green and finally to a peacock-blue. The latter appears to be remarkably stable for many hours, in spite of the theoretical development of colorless oxidation products. At the end of 18 hours, the colors of the standard and unknown solutions are matched in a colorimeter and the usual colorimetric calculations applied.

In Table I are recorded the results of a comparative study of the van den Bergh (diazo) and oxidation tests. In this case, fresh bile was obtained daily from a drainage tube inserted in the common bile duct following cholecystectomy. Analyses were made daily for 1 week. It will be observed that, with one exception, less than one-half of the total bile pigments was accounted for by the van den Bergh procedure. (Incidentally, as all analyses were made upon *fresh* bile, the data give some idea of the possible degrees of oxidation of bile pigments within the hepatic system.)

These data, of course, merely indicate that all of the bile pigments are not accounted for by the diazo reaction. They obviously give no accurate idea of the degree of specificity of the diazo reagent with respect to bilirubin. Further investigation is necessary. The next step, therefore, was to expose bile to ordinary

[2] The bilirubin used in this work was prepared by the Eastman Kodak Company and is believed to be very pure. It was crystalline, had a bright orange color, was completely, though slowly, soluble in chloroform, and left no ash whatever on heating.

As in all colorimetric work, the intensity of the standard should approximate that of the unknown solution. Standard solutions should, therefore, be prepared ranging between 0.1 and 0.25 mg. of bilirubin per cc. Solutions containing as little as 0.1 mg. of bilirubin per 1 cc. of chloroform are quite stable, as both Hunter (2) and Fischer and Barrenscheen (1) have shown. Weaker solutions tend to undergo oxidation rapidly. A solution containing 0.01 mg. per cc., normally light golden yellow, becomes almost colorless at the end of 24 hours when exposed to light and then gives a very slight color reaction only with the oxidizing reagent. Standard solutions should, therefore, be stored in dark glass bottles.

room air. Following such exposure, one would expect that the bilirubin, as well as the other pigments, would, in time, undergo further oxidation. Should the diazo reaction be specific for bilirubin, one would then expect, in time, to find little or no color development with this reagent, though the test for total bile pigments would still yield appreciable color.

The results obtained following exposure of bile to room air are shown in Table II. The data, interpreted on the basis of the above observations, indicate a fairly high degree of specificity of the diazo reagent for bilirubin. It will be observed that, in time, though the van den Bergh reaction gave practically no indication

TABLE I

Bilirubin Compared With Total Pigment Content of Bile

Showing That Ehrlich's Diazo Reaction Is Probably Highly Specific for Bilirubin

Subject 1037-32.

Day of experiment	Pigment, mg. per 100 cc. bile	
	Van den Bergh method	Oxidation method
1	37	89
2	38	90
3	32	80
4	30	84
5	31	74
6	32	80
7	37	64

of the presence of bile pigments, there was, relatively, an inappreciable decrease of total pigment. Andrewes (12) first noted that though bile when fresh gives the "direct" type reaction with the diazo reagent, when exposed to air it gives, in time, the "delayed" type reaction. This has been confirmed repeatedly. The possibility of this phenomenon explaining the above results is, however, excluded, since the quantitative van den Bergh reaction includes all pigments; both the direct and delayed types of bilirubin couple with the diazo reagent in alcohol.

Assuming the van den Bergh procedure to be sufficiently quantitative and specific for renal threshold studies, there is another variable to consider; namely, the different behavior of sera towards

the diazo reaction. Many hypotheses have been advanced to explain the latter. Whether these differences are due to combination of pigments with protein (13, 14) or lipids (15), to an ammonium salt of bilirubin (16), or sodium hydrogen bilirubinate (2, 8) is, as yet, problematical. Important from the point of view of renal threshold studies is the fact that there appear to be at least two different types of pigments. The rate of coupling with diazo compounds in media of different pH values, the reaction to oxidizing agents, and solubility and dialyzing properties afford proof.

TABLE II

Showing That Ehrlich's Diazo Reaction Is Highly Specific for Bilirubin; That As Bilirubin Disappears From Bile, Color Given by Reagent Decreases in Intensity

Experiment No.	Pigment, mg. per 100 cc. bile					
	Fresh bile		After 24 hrs.		After 72 hrs.	
	Total pigment (oxidation method)	Van den Bergh method	Total pigment (oxidation method)	Van den Bergh method	Total pigment (oxidation method)	Van den Bergh method*
1	74	40	70	11	64	Trace
2	69	36	66	13	65	"
3	96	30	91	18	79	"
4	116	36	109	19	94	"

* Quantitative estimations were not possible, because of differences in color between the standard and bile solutions.

The observation that there are at least two different types of pigments is important, since, if the "delayed bilirubin" does not dialyze readily and the "direct bilirubin" does (17–19), it would appear that the direct type is more likely to account for the bilirubin in the urine. Therefore, measurement of *total* bilirubin alone would give no indication of the amount of pigment available for excretion. Proof of this is afforded in hemolytic jaundice. In this condition, though the urine contains no bilirubin, the blood may contain as much as 10 units or more. McNee (20) reports a case showing 11 units, and Rosenthal and Holzer (21) record a value as high as 18 units. Beaumont and Dodds (22) suggest that under these conditions the bilirubin is probably excreted in some

other form (urobilin?). However, the possible high concentration of bilirubin in the blood with no bilirubin in the urine, in the absence of obvious renal disease, clearly indicates that some factor other than renal function governs the urinary excretion of this pigment. This condition might be regarded as an exceptional case and excluded from renal threshold work. There is, however, the further difficulty in that the delayed reaction type of pigment, characteristic of this disease, is found in practically all forms of jaundice. As a matter of fact, except for *early* obstructive jaundice, it appears to be universal, since obstruction, if of long duration, leads to hepatitis (23), a condition in which the delayed type of pigment usually dominates.

In view of the number of difficulties which surround any attempt to determine whether bilirubin is a threshold substance, the basis for the generally accepted belief that it is seems worthy of investigation. In an early review, McNee (20) records his own experiences and those of van den Bergh and Lepehne. All three observers are agreed "that bile pigment appears in the urine whenever the content of the serum reaches about 4 units. . . . Below this figure, biliuria is absent. . . ." As far as could be ascertained, these early observations alone form the basis of the statements found widely in the literature and text-books. The original observations of van den Bergh, Lepehne, and McNee are, as we believe our data here indicate, due to the unsatisfactory methods then available for detection of bilirubin in urine. Because of these methods, clinicians, as is well known, tended to rely upon the color of the skin and mucous membranes rather than upon laboratory methods. In 1930, Hunter (7) introduced his method for detecting bilirubin in urine and our experiences with the test confirm its sensitivity. Though we have found that bilirubin, when added to urine, could not be detected by this method in as low concentrations as when in simple solution or in blood, the test was found to be much more sensitive than the many available heretofore. With this method, bilirubin added to urine could be detected when present in as high a dilution as 1:666,000. The following illustrates an experiment.

A solution of bilirubin in chloroform was made so that 1 cc. contained 0.25 mg. of pigment. Different amounts of this solution, ranging between 0.1 and 1.0 cc., that is 0.025 to 0.25 mg. of

bilirubin, were then placed in a series of beakers and the chloroform was allowed to evaporate. The bilirubin residue was brought into solution with the aid of sodium hydroxide and transferred quantitatively to 100 cc. volumetric flasks, first with the aid of a few cc. of water and then with urine. The solutions were then diluted to the 100 cc. mark with urine. The flasks were well shaken and the contents tested for bilirubin according to Hunter's technique, with the following result.

Flask No.	Bilirubin	Dilution	Hunter's test
	mg. per 100 cc.		
1	0.025	1:4,000,000	Negative
2	0.050	1:2,000,000	"
3	0.075	1:1,333,000	"
4	0.100	1:1,000,000	"
5	0.125	1:800,000	"
6	0.150	1:666,000	Positive
7	0.175	1:570,000	"
8	0.200	1:500,000	"
9	0.225	1:444,000	"
10	0.250	1:400,000	"

It will be observed that Flask 6 which contained as little as 0.15 mg. of bilirubin per 100 cc. of urine (1:666,000 dilution) gave a positive reaction. The fact, however, that bilirubin can be detected in simple solution and even in blood when present in as high a dilution as 1:1,000,000 or more, clearly indicates that, sensitive as Hunter's test may be, it still lacks that sensitivity necessary to state definitely whether bilirubin is, or is not, a normal constituent of urine. This observation has, as shall be shown, an important bearing upon the interpretation of the data of the following experiments.

Selection of Subjects for Investigation

In this study, blood and urine analyses were made in cases of jaundice. Blood and urine analyses are a routine in every case of jaundice admitted to this hospital and the frequency of such analyses depends upon the clinical condition. As daily observations are of quite frequent occurrence, it was a simple matter to determine in practically every case of decreasing jaundice when the

patients became suitable subjects for this study; that is, when the bilirubin content of the blood approached the generally accepted renal threshold level (4 units). In addition, a large amount of data was available from individuals with early and increasing jaundice.

From experiences with many thousands of analyses, it was observed that, if we exclude rapid destruction of liver tissue (*e.g.* acute yellow atrophy) and sudden relief, a mechanical obstruction to the outflow of bile (*e.g.* removal of stone in common bile duct), the concentration of bilirubin in blood neither rises nor falls very rapidly; as a rule, both the onset and recovery of jaundice are relatively slow and the concentration of bilirubin in blood is remarkably constant over a period of hours. This phenomenon tends to simplify renal threshold studies, since, as is well known, one of the greatest obstacles in such work is the uncertainty that any given sample of urine corresponds to a given blood concentration.

A summary of the results of over 100 experiments is given in Table III. All results are expressed in terms of mg. of bilirubin per 100 cc. of blood. The purpose of this was to insure proper interpretation of the data, in view of the uncertainty involved in the use of the unit. Hunter (2) first called attention to an obvious, but apparently overlooked, error in the original calculation of the unit. Judging from more recent literature and text-books, this error appears again to have been overlooked.

The color of the original artificial standard which was meant to match that produced by 1 unit of bilirubin corresponded to that obtained with a mixture of 10 cc. of alcohol, 2.5 cc. of diazo reagent, and 0.05 mg. of bilirubin. Since 12.5 cc. of solution contained 0.05 mg. of bilirubin, the true unit corresponds to a 1:250,000 and not to a 1:200,000 dilution of bilirubin. Expressed more exactly, the true unit corresponds to 0.4 and not 0.5 mg. of bilirubin per 100 cc. of blood.

The data of this frequency distribution (Table III) clearly indicate that bilirubin is excreted in the urine when the concentration of this pigment in the blood is at a much lower level than the generally accepted threshold. As a matter of fact, as some of the data in Table III show, bilirubin was found in the urine with as low a concentration of blood bilirubin as 0.12 mg.

per 100 cc.   As such amounts of bilirubin are regarded as normal, the concept of a renal threshold for this blood constituent does not appear to be tenable.

The fact that bilirubin is not found in the blood in a concentration of 0.12 mg. per 100 cc. under normal conditions suggests that the kidneys may behave towards bilirubin as they do towards glucose. This phenomenon will again be referred to.

Since nephritis is known to affect the renal threshold for glucose and as kidney damage very commonly accompanies and may actually be caused by jaundice (due probably to the toxic properties of bile salts), the urines were also examined for albumin and casts. The cases were then divided into (a) those with, and (b) those

TABLE III

Relationship between Concentration of Bilirubin in Blood and Presence of Bilirubin in Urine

Blood bilirubin	No. of tests	No. showing bilirubinuria
mg. per 100 cc.		
0.08–0.2	29	9
0.21–0.4	38	19
0.41–0.6	9	4
0.61–0.8	12	7
0.81–1.0	2	1
1.01–1.2	6	4
1.21–1.4	0	0
1.41–1.6	3	3
1.61	10	10

without, albuminuria.   A summary of the results is shown in Table IV.   It will be observed that there was no relationship between albuminuria and bilirubinuria.

An interesting observation was that the lowest values of blood bilirubin at which bilirubin was found in the urine were observed in individuals in whom the jaundice was *decreasing*.   The results of six such cases are shown in Table V.   Frank (24) first made the observation that sugar appears in the urine at a much lower level of blood sugar concentration when the sugar content of the blood is decreasing than when it is increasing.   It was because of the quite frequent occurrence of this phenomenon that one of the writers (I.M.R.) called attention to the unreliability of blood sugar

time curves, when used alone, for the diagnosis of renal glycosuria
(25). This similarity of behavior of the kidneys towards bilirubin
and towards glucose and the fact that we have not been able to
detect bilirubin in normal urine suggest that this pigment may be a

TABLE IV

Absence of Relationship between Albuminuria and Bilirubinuria

Blood bilirubin	Albuminuria		No albuminuria	
	No. of tests	No. showing bilirubinuria	No. of tests	No. showing bilirubinuria
mg. per 100 cc.				
0.08–0.2	11	4	18	5
0.21–0.4	23	9	15	10
0.41–0.6	2	1	7	3
0.61–0.8	7	4	5	3
0.81–1.0	1	1	1	0
1.01–1.2	2	2	4	2
1.21–1.4	0	0	0	0
1.41–1.6	2	2	1	1
1.61–1.8	4	4	6	6

TABLE V

*Concentrations of Bilirubin in Blood at Which Bilirubin Was Found in Urine
in Individuals with Decreasing Jaundice*

Bilirubin, mg. per 100 cc. blood

Subject No................	214-32	182-32	184-32	6943-31	7422-31	6125-31
Day of experiment						
1	1.6	2.4	1.6	1.6	0.6	1.2
2	1.2	0.8	1.3	0.4	0.1	1.0
3	0.4	0.4	0.1	0.4		0.6
4	0.4	0.2		0.2		
5	0.4	0.1		0.3		
6	0.3			0.2		
7	0.2					
8	0.2					

threshold substance. There is, however, the fact that bilirubin
was also found in the urine, with low concentrations of blood
bilirubin when the jaundice was increasing. Observations were
made in Subjects 184–32 and 6943–31 both during increasing and

decreasing jaundice and, during the former, bilirubin was found in the urine with as little as 0.2 and 0.25 mg. of bilirubin per 100 cc. of blood. In another case of increasing jaundice (Subject 374-32), it was found with 0.6 mg. The failure to detect bilirubin in the urine of normal individuals may also be due to the method employed, rather than to a renal threshold. As stated, sensitive as Hunter's method may be, compared with other procedures for the detection of bilirubin, it does not detect this pigment when it is added to urine in as low concentrations as when in simple solution.

SUMMARY

In order to determine whether the renal threshold concept applies to bilirubin, samples of blood and urine were collected simultaneously and analyses were made with respect to their bilirubin content.

The data clearly indicate, if bilirubin is at all a renal threshold substance, that the threshold value is very much below that generally accepted, and that the idea that bilirubinuria is absent below 4 units of blood bilirubin does not hold.

No relationship was found between albuminuria and bilirubinuria.

Other than the fact that bilirubin could not be detected in normal urine by the method employed, the combined data of this preliminary study fail to support the generally accepted view that there is a renal threshold for bilirubin.

Because of the different behavior of the test towards bilirubin in urine and in simple solution, it is suggested that with a still more sensitive method for detecting bilirubin this pigment may be found to be a normal constituent of urine.

Attention is again called to an error made originally in the calculation of the unit of bilirubin.

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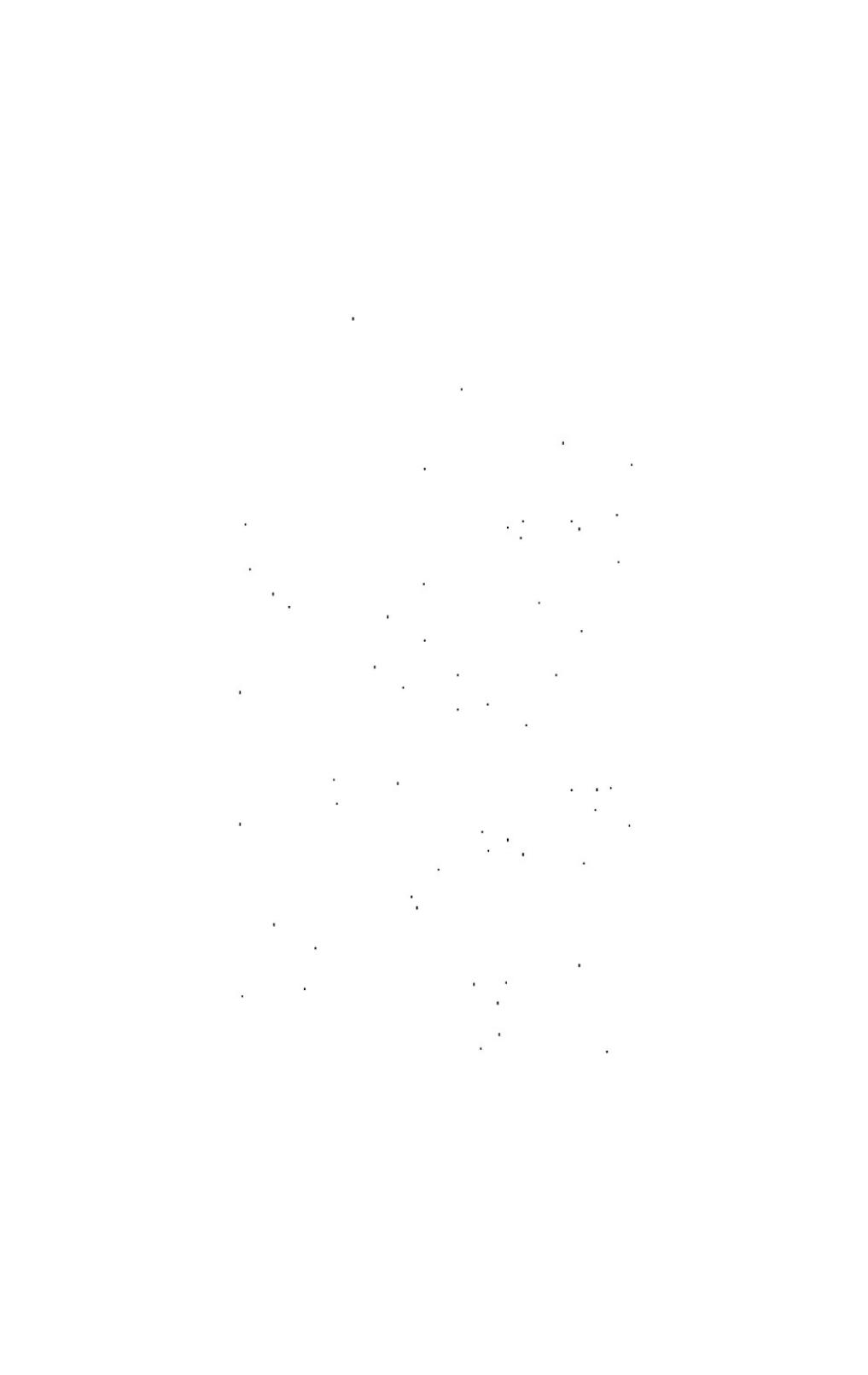
THE FORMATION OF METHYLGLYOXAL BY CLOS-
TRIDIUM ACETOBUTYLICUM

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Although considerable progress has been made toward an understanding of the later phases of the fermentation of carbohydrates by *Clostridium acetobutylicum*, very little is known regarding the very early stages of sugar breakdown in this organism. The first systematic attempts to identify the substances intermediate between carbohydrate and the neutral end-products of the fermentation were those of Speakman (1) and of Reilly *et al.* (2). It was clearly shown that acetic and butyric acids accumulate in large amounts during the first half of the fermentation. Together they represent practically all of the volatile acid formed, with the exception of very small amounts of formic acid which were reported by Stiles, Peterson, and Fred (3); the accumulation of volatile fatty acid parallels the disappearance of sugar up to the time of maximal acidity, after which the amounts of the acids present at any moment represent a balance between the quantities produced from sugar and those removed by conversion into neutral end-products.

The nature of the primary reactions leading to the formation of the two main volatile acids from glucose is not clearly understood. The suggestion that the 6-carbon compound undergoes direct cleavage into the 2-carbon and 4-carbon compounds, acetic and butyric acids, is open to serious criticism on several grounds, not the least important of which is the failure to explain the gas relationships of the fermentation. The fact that the gas evolved in the very earliest stage of the fermentation of glucose is probably pure hydrogen suggests very strongly that pyruvic acid is a primary degradation product, but there is no positive evidence

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in support of this hypothesis. Moreover, the observation of Wilson, Peterson, and Fred (4) that acetylmethylcarbinol is a regular end-product indicates that acetaldehyde is probably present at some stage although, as Lafon (5) has recently suggested in the case of *Bacillus subtilis*, acetylmethylcarbinol may possibly be formed directly from a 4-carbon compound rather than from acetaldehyde. In any case numerous factors enter into a consideration of the origin and rôle of acetaldehyde and a proper evaluation of these is outside the limits of the present discussion. It is the purpose of this paper to present evidence that methylglyoxal is formed as the result of the action of the organism on hexosephosphate and to suggest that this ketoaldehyde is a primary product of the degradation of glucose in the normal fermentation.

Since the observation of Pinkus (6) that methylglyoxal is formed during the decomposition of sugars in alkaline solution, frequent suggestions have been made that it is present as a transitory substance in glycolysis in biological systems. Until recently most of the evidence in support of these suggestions was of an indirect type based upon observations of the behavior of cells and tissues toward methylglyoxal and upon attempts to identify the products of the action, if any, with substances formed during the course of normal metabolism. Such methods are valuable in strengthening the line of evidence but in themselves do not, of course, provide proof of the ability of the organism or tissue to accomplish the formation of methylglyoxal. It is therefore desirable, if possible, so to establish conditions that the compound, if formed from glucose or related substance, is not wholly transformed at once but is allowed to accumulate in sufficiently large amounts to make possible its isolation and identification. The earliest report of the isolation of methylglyoxal formed biologically appears to be that of Fernbach (7) who observed its formation through the action of *Tyrothrix tenuis* on glycerol and isolated it as the osazone having the characteristic melting point.

More recently numerous investigations have established its formation by various animal and plant tissues; in practically all cases the sugar employed was in the form of hexosephosphate. Toenniessen and Fischer (8) in 1926 first isolated methylglyoxal

as a product of glycolysis in animal tissues, using the pancreas and muscles of pigs. Since then it has been shown to be formed by muscle and liver of rabbits (9), by blood (10), by liver, kidney, and muscle of pigs and rabbits (11), by human erythrocytes and leucocyctes (12), by frog and rabbit muscle (13), by rat sarcoma (14), and by pike muscle (15). Its formation has also been demonstrated through the action of tobacco leaves (16) and germinating peas and beans (17) on hexosephosphate.

Several species of microorganisms have recently been shown to possess the ability to transform hexosephosphates, at least partially, into methylglyoxal. The latter was included in 1920 in a scheme representing the alcoholic fermentation of glucose (18), but it was not until 1928 that Neuberg and Kobel (19) identified pyruvic aldehyde as a product of the action of *Saccharomyces cerevisiæ* on hexosediphosphate, the compound having been isolated as the bis-2,4-dinitrophenylhydrazone. In the previous year Kostytschew and Soldatenkov (20) reported its isolation as an apparent product of the action of yeast on sucrose but the substance isolated was shown (21) to be a decomposition product of the semicarbazide reagent employed. Other workers have confirmed the results of Neuberg and Kobel with *Saccharomyces cerevisiæ* (22) and have extended the observations to other yeasts, including *Torula colliculosa* (23) and *Saccharomyces johannisberg* (24). At least five bacterial species have been shown to form methyglyoxal from hexosephosphate; namely, *Bacillus delbrücki* (25), *Bacterium coli* (26), *Bacillus lactis aerogenes* (27), and the tubercle and timothy bacilli (28). In all cases either dried organisms, plasmolyzed cells, or extracts were employed. The fact that all of these organisms possess the enzyme system responsible for the formation of methylglyoxal from hexosephosphate is particularly significant in view of the rather wide diversity of substances which are formed by the organisms as normal metabolic products.

The products of the normal activity of *Clostridium acetobutylicum* are quite different from those associated with the above organisms and with normal muscle. It is therefore of considerable interest to observe that in the case of this organism also, methylglyoxal is readily formed from hexosephosphate.

Enzyme Preparation—The organisms were grown in a medium containing 4 per cent glucose, 1 per cent Bacto-Peptone, 0.5 per cent KH_2PO_4, 0.5 per cent K_2HPO_4, and shredded filter paper. After 24 hours incubation at 37.5° the culture was filtered through cheese-cloth and centrifuged. After several washings the organisms were dried rapidly in a current of air at room temperature. After further drying in the desiccator they were ground to a fine powder.

Substrate—Magnesium hexosephosphate was prepared from a commercial sample of calcium hexosephosphate[1] somewhat after the manner of Neuberg and Sabetay (29). An analysis of this magnesium salt revealed only traces of inorganic phosphate and indicated, moreover, that, although it was chiefly the diphosphate ester, small amounts of the monophosphate ester were still present.

Conditions—pH 6.0 was maintained by 0.02 M phthalate buffer at a temperature of 37.5°. All flasks were rocked gently in the water bath in order to keep the organisms uniformly suspended.

Experimental Flask—This contained 500 cc. of 1 per cent magnesium hexosephosphate, 0.02 M with respect to phthalate buffer; 12.0 gm. of dried bacteria; and 10 cc. of toluene.

Control Flask I—Flask I contained 10 cc. of 0.02 M phthalate buffer, 0.24 gm. of dried bacteria, and 0.5 cc. of toluene.

Control Flask II—Flask II contained 10 cc. of 1 per cent magnesium hexosephosphate, 0.02 M with respect to phthalate buffer, and 0.5 cc. of toluene.

After 48 hours sufficient trichloroacetic acid was added to the experimental flask to make a 4 per cent solution, and after standing for 1 hour the mixture was centrifuged. To the clear solution were added 150 cc. of a saturated solution of 2,4-dinitrophenyl hydrazine in 2 N HCl. After standing 3 hours the mixture was centrifuged; the orange-colored precipitate was washed successively with 2 N HCl, water, 25 per cent Na_2CO_3 until the washings were no longer brown, water again, and finally ethyl alcohol. The precipitate was dried in a vacuum desiccator at 110°, dissolved in nitrobenzene, and reprecipitated. The substance gave the blue

color with alcoholic KOH described by Neuberg and Kobel (30) as characteristic of methylglyoxal-bis-2,4-dinitrophenylhydrazone. Melting point and analytical data are as follows:

Observed m.p. 298° (corrected) for methylglyoxal-bis-dinitrophenyl-hydrazone.

		Carbon per cent	Hydrogen per cent	Nitrogen per cent
$C_{15}H_{12}N_8O_8$.	Calculated.	41.66	2.80	25.93
	Found.	41.61	2.74	25.88

Vacuum Sublimation of Methylglyoxal-Bis-Dinitrophenylhydrazone—If the substance, either before or after treatment with nitrobenzene, be subjected to sublimation at 10 mm. pressure and 220°, orange-colored, needle-like crystals are formed in a few minutes. Crystals obtained in this manner gave the characteristic blue color reaction and, when observed under the microscope in a micro melting point apparatus, were found to melt sharply at 297.5° (corrected).

Possible Formation of Pyruvic Acid

Upon acidifying the brown sodium carbonate washings from the methylglyoxal-bis-dinitrophenylhydrazone a small quantity of a pale yellow precipitate was obtained. After having been redissolved in carbonate and reprecipitated several times this substance gave the red-brown color test (30) with alcoholic KOH. This is regarded as a preliminary indication of pyruvic acid as a later product of the fermentation of hexosephosphate by this organism. This color reaction has been obtained so frequently at this stage in numerous small experiments that the formation of pyruvic acid may be regarded as definitely probable. However, confirmation by isolation and analysis is necessary.

SUMMARY

1. Methylglyoxal, isolated and identified as the bis-2,4-dinitro-phenylhydrazone, has been shown to be a product of the action of dried cells of *Clostridium acetobutylicum* upon magnesium hexose-phosphate.

2. It is suggested that this ketoaldehyde is an early transitory product of the normal metabolism of carbohydrate in this species.

3. A preliminary indication of pyruvic acid as a further product

of the fermentation of hexosephosphate by this organism is reported.

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FURTHER OBSERVATIONS ON THE NATURE OF THE HIGHLY UNSATURATED FATTY ACIDS OF BEEF BRAINS*

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Previous reports (1, 2) have shown that there occurs in beef brains fatty acids more highly unsaturated and of higher molecular weight than arachidonic acid, $C_{20}H_{32}O_2$. The presence of tetracosapentenoic acid, $C_{24}H_{38}O_2$, has been suggested. Although a number of preparations of highly unsaturated acids were made and analyzed, the amounts prepared were always too small to warrant final conclusions. In the present investigation the problem of the nature of these acids has been further clarified by the preparation of somewhat larger quantities. Thus, it has been possible fractionally to distil the methyl esters of the acids, to analyze the fractions, and, in one instance, to reduce one of the fractions for further identification of the saturated acid. The results are surprising. Instead of the acids consisting mainly of a mixture of arachidonic and tetracosapentenoic acids, they have been found to consist chiefly of acids of the C_{22} series, docosatetrenoic and docosapentenoic acids. There is also the possibility of the presence of small amounts of tetracosapentenoic acid.

EXPERIMENTAL

At present the best method available for the preparation of highly unsaturated acids is to isolate and reduce the ether-insoluble bromides. Since brain tissue yields less than 1 per cent of its weight of bromides, it is clear that large quantities of the tissue must be worked up. The alkaline hydrolysis-butyl alcohol method described previously (2) is well adapted for this purpose.

Briefly, the method employed was as follows: 5 kilos of fresh beef brains were hashed. 1 kilo each was transferred to three Pyrex 5 liter flasks. 2 kilos were placed in a 12 liter balloon flask. 50 per cent NaOH was added to each in the proportion of 400 cc. to each kilo of tissue. The mixtures were cautiously brought to the boiling point with occasional stirring. When liquefied (5 to 10 minutes), they were combined in the 12 liter flask and the boiling continued slowly for 1 to 2 hours. An atmosphere of natural gas from the laboratory lines was used to prevent oxidation. Upon completion of the hydrolysis the mixture was allowed to cool for 10 minutes, when 2 liters of butyl alcohol were added. After refluxing for 10 minutes, the alcohol was allowed to separate. The lower aqueous alkaline layer was withdrawn by siphon and discarded. An excess of HCl was added to the alcohol and shaken, following which the flask was filled with water (total volume 12 liters). After separation into two layers, the water was removed as nearly quantitatively as possible by siphon, and the butyl alcohol solution of fatty acids, cholesterol, and many other substances was transferred to a 5 liter flask and distilled under reduced pressure. During the early parts of the distillation the water came over with the butyl alcohol, thus serving to dehydrate the fatty acids. The process was hastened and the pressure and temperature kept low to minimize formation of butyl esters. When most of the alcohol had come over, ether was added slowly with constant shaking until the flask was nearly half full. The solution was set aside until another run of 5 kilos of brains could be brought to this point, when the two runs, corresponding to about 25 pounds of tissue, could be combined in the one 5 liter flask. After standing in an ice box overnight, the ether solution was filtered from the precipitated material, and placed in an ice-salt mixture and brominated slowly, an excess of bromine being used. The bromides were recovered and washed by repeated decantation, by placing in a tall specimen jar holding 5 liters. After standing a day in the cold, the bromides had settled about four-fifths down the jar, so that about this part of the clear ether layer could be removed by siphon. Fresh cold ether was added, and the separation repeated five times. After the last washing, the bromide-ether suspension was transferred to 250 cc. centrifuge bottles, the final separation being carried out by centrifugation.

The bromides were dried and analyzed. They were light brown, nearly white in color. The results of four runs (approximately 100 pounds) are given in Table I. The average polybromide number was 0.92 which is slightly higher than was reported previously (0.88) (2).

333 gm. of the bromides were treated with zinc dust in boiling dry methyl alcohol, in an atmosphere of CO_2 for a day. After removal of zinc and zinc bromide the alcoholic solution was treated with dry HCl and further refluxed for 5 hours to assure complete esterification. The esters were recovered and distilled. The product was a light amber oil, weighing 38 gm. (yield 35 per cent), boiling at 210–248° at 5 mm. pressure. The wide range of boiling point was due to the rapid distillation. The iodine number was 344.2.

TABLE I

Yield of Polybromides from Beef Brains

Lot No.	Weight of brains	Weight of bromides	Polybromide No.	Per cent Br
	gm.	*gm.*		
1	10,800	104.0	0.96	63.32
2	9,585	88.5	0.92	58.38
3	11,150	95.1	0.85	64.94
4	9,550	89.7	0.94	58.76

After analysis, 30 gm. of the esters were fractionally distilled, the results being given in Table II.

The bromides in Fractions 1 and 2 partly melted with decomposition between 250–260°; those from the other fractions shrank to a black mass without melting. No sintering at the 230° level would indicate the absence of appreciable quantities of methyl octobromoarachidate which melts at that point. It is possible that Fractions 1 and 2 contain some methyl docosatetrenoate, the octobromide of which should melt near 250°.

9 gm. of Fraction 2 were reduced in the Burgess-Parr catalytic apparatus. The reaction at 40 pounds pressure in alcohol, heated to 60°, was complete in 5 minutes. The iodine number fell from 346.7 to 4.4. The product was a white solid melting at 47°. This was distilled, saponified, and the free acid liberated and crystallized from acetone. The first crop of crystals melted at

77° (uncorrected) and gave a molecular weight by titration of 331.6. After eight crystallizations the product consisted of beautiful flat shining blades, melting at 78.5–78.7° (corrected). The reported melting point for behenic acid is 80–82°. The molecular weight as determined was 339.4 (theory for behenic acid, 340).

TABLE II

Results of Fractionation and Analyses of Methyl Esters of Highly Unsaturated Fatty Acids of Beef Brains

Fraction No.	Weight	Boiling point	Iodine No.	Mean molecular weight of acids*	Polybromide No.	Per cent Br
	gm.	*°C.*				
1†	6.0	190–200	331.6	332.1	90.38	67.62
2	13.2	200–205	346.7	335.6	97.7	68.54
3	7.4	205–210	358.1	333.7	111.3	69.25
4†	2.5	210–217	354.6	343.6	96.0	67.38

* By subtracting 14 from the molecular weight of the esters.

† Nearly all of Fraction 1 came over slightly below 200° and Fraction 4 slightly over 210°.

DISCUSSION

The analytical data in Table II may be summarized as follows: Most of the product boiled within the range 200–210°. If one allows for the time necessary for the distilling apparatus to warm up to an even temperature, and the small amount of material distilled, this amounts almost to a constant boiling product. The molecular weight of the acids confirms this observation, that of the first three fractions being almost constant, with an appreciable rise for Fraction 4. The average for the first three is 333.8. The molecular weight of docosapentenoic acid is 330. The esters, therefore, are essentially those of the C_{22} series. The possibility of the presence of small amounts of C_{20} and C_{24} acids is not precluded by these results.

The iodine numbers of the esters range from 331 to 358. The theory for methyl docosatetrenoate is 293 while that for the docosapentenoate is 369. It is apparent that the 5-bond acid largely predominates. The bromide from the ester of this acid, methyl decabromodocosanoate (behenate) should contain 69.9

per cent bromine. The bromides from Fractions 2 and 3 are in fair agreement with the theory. The polybromide numbers offer no further critique of identification.

The mean molecular weight of the reduced acids from Fraction 2 is only slightly low for behenic acid. After repeated crystallizations from acetone the molecular weight is in excellent agreement with the theory. The melting point of the purified product is slightly lower than that reported for behenic acid.

The data here presented show no evidence for the presence of ordinary arachidonic acid, such as occurs in the glandular lipids. There is, in fact, little or no evidence for acids below the C_{22} series.

The possibility of the presence of small quantities of tetracosapentenoic acid is somewhat greater. Fraction 4 showed a significant rise in molecular weight and a sudden sharp rise in boiling point at the end of the distillation, which may have been due to the presence of this acid.

These results warrant the conclusion that the highly unsaturated fatty acids of beef brains, prepared as described above, belong chiefly to the C_{22} series.

SUMMARY

1. The methyl esters of the highly unsaturated fatty acids from approximately 100 pounds of beef brains have been prepared and fractionally distilled.

2. The acids belonged chiefly to the C_{22} series. Docosapentenoic was the predominant acid present, along with small amounts of docosatetrenoic acid.

3. The presence of arachidonic acid such as occurs in the glandular tissues could not be demonstrated.

4. There is a possibility that small amounts of tetracosapentenoic acid may have been present.

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THE FREEZING POINTS OF SERUM AND CORPUSCLES

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The question of the equality of the osmotic pressure of the fluid within the red blood cells with that of the serum surrounding them is of fundamental importance. Van Slyke, Wu, and McLean (1) have postulated equality, and on this basis have made important mathematical deductions concerning water and electrolyte distribution in blood and concerning the manner in which this distribution is altered by changes in carbon dioxide and oxygen tensions.

Certain experimental evidence seems to indicate that the osmotic pressures are not equal. A number of investigators (Stewart (2), Moore and Roaf (3), Hamburger (4), Collip (5), Ege (6), Cherbuliez (7), and Koeppe (8)) have found the freezing point depression of the corpuscles to be less than that of the serum. Their average difference is 0.042°. Since the freezing point depression is a measure of osmotic pressure, these results mean that the osmotic pressure of the corpuscles is less than that of the serum. The above difference, in terms of osmotic pressure, corresponds roughly to 400 mm. of Hg. Since there are many observations indicating that red blood cells behave as simple osmometers (Lucké and McCutcheon (9)), it seems rather strange that such a difference should exist between the osmotic pressures of the serum and corpuscles. For this reason the present investigations were undertaken.

The experimental procedures followed by these workers are very similar. All except Ege used defibrinated blood. The serum and corpuscles were separated by centrifugation (except in the case of Koeppe, who allowed the blood to stand for a day and then used the supernatant serum and sedimented corpuscles). The freezing points were determined by the Beckmann method.

The work of Kroenig and Fueth (10) should be mentioned. As

far as we can find, these investigators are the only ones who have found the freezing points of cells and serum to be identical. Their experimental procedure differs from that of others in the manner of separating cells from serum. The blood (human) was not defibrinated; but, instead, it was allowed to clot, and kept in the ice box for a period varying from about $4\frac{1}{2}$ hours to 6 days until the serum had been squeezed out leaving a firm clot. The serum was pipetted off; the blood clot was put on a filter cloth in a funnel, and the fluid worked through with a platinum loop. The freezing point determinations were made on these fluids. It is significant that Kroenig and Fueth did not centrifuge their blood in the separation of serum from corpuscles, while all the other investigators listed above did so (with the exception of Koeppe in his one determination).

Method

The Beckmann apparatus was used in the determination of the freezing points. There is a good discussion of the method by Biltz (11). Since this technique is a standard laboratory procedure, it will not be discussed at great length. The tube containing the blood, which was of 2 cm. diameter and which was provided with a side tube, was placed in a larger, thick walled tube of 3 cm. diameter. The temperature of the ice-salt freezing mixture was not allowed to fall more than about 3–4° below the freezing point of the blood. The thermometer was kept in an upright position in an ice and distilled water mixture in a vacuum bottle. It is said (Biltz (11) and Auwers (12)) that the elastic conditions of the glass corresponding to a low temperature do not become established immediately. The thermometer was tapped at the top before a reading was taken in order to avoid errors due to sticking of the mercury thread. Undercooling was limited as described in the next section. All determinations were made in duplicate on different samples.

Defibrinated blood was used in all experiments. Beef blood was obtained from the abattoir. The animals were first rendered unconscious by a blow on the head; and, after a time, the throat was cut, and the blood collected. Since the blow on the head paralyzed respiration, the blood was very venous in character. The blood in the case of the dog was drawn by arterial (femoral)

puncture from the unanesthetized animal. It was then defibrin-
ated. This blood was arterial. None of the blood used in these
experiments was taken from anesthetized animals. Ether or
chloroform narcosis raises the osmotic pressure of the blood
(Carlson and Luckhardt (13)).

The serum was separated from the corpuscles by centrifugation
for $\frac{1}{2}$ to 1 hour. After centrifuging, as much as possible of the
serum was pipetted off. There is always a layer of serum above
the corpuscles which it is impossible to remove. In order to
secure corpuscles as free as possible from serum, a pipette was
introduced with its tip at the bottom of the tube. The pipette
was then filled. In this way only the bottom layers were obtained,
and the top layer with the serum was eliminated.

In working with corpuscles and serum certain factors, which
may cause errors, must be considered. These are: amount of
undercooling and the water content of the material, evaporation
in centrifuging, escape of carbon dioxide, temperature of cen-
trifuging.

Each of these will be considered in detail in some part of the
paper.

Correction for Undercooling

It is necessary to correct the freezing point reading for under-
cooling. The correction may be made by the formula, $\Delta = \Delta' -$
$\dfrac{su\,\Delta'}{l}$ (Harris and Gortner (14)), Δ being the true freezing point,
Δ' the observed freezing point, s the specific heat of the material,
u the undercooling, and l the latent heat of fusion of ice. This
equation is based on the assumption that the material is almost
entirely water with only a negligible amount of solid. However,
if there is a large amount of solid, the effect of undercooling on
the observed freezing point is magnified. The error from under-
cooling comes from a concentration resulting from the ice forma-
tion, the heat from which is necessary to raise the temperature
from the undercooled temperature to the observed freezing point.
For a given undercooling, assuming the specific heats to be unity,
there will always be formed the same quantity of ice regardless of
the water content of the material. Hence, the concentration
will be greater in materials of low water content because the same

amount of ice has now been removed from a smaller amount of solution. The equation, $\Delta = \Delta' - \dfrac{su\,\Delta'}{80\,w}$ (Collins (15)), takes account of the solids, w representing the fraction by weight of water acting as a solvent and l having been set equal to 80. In this paper s has been assumed to be unity.
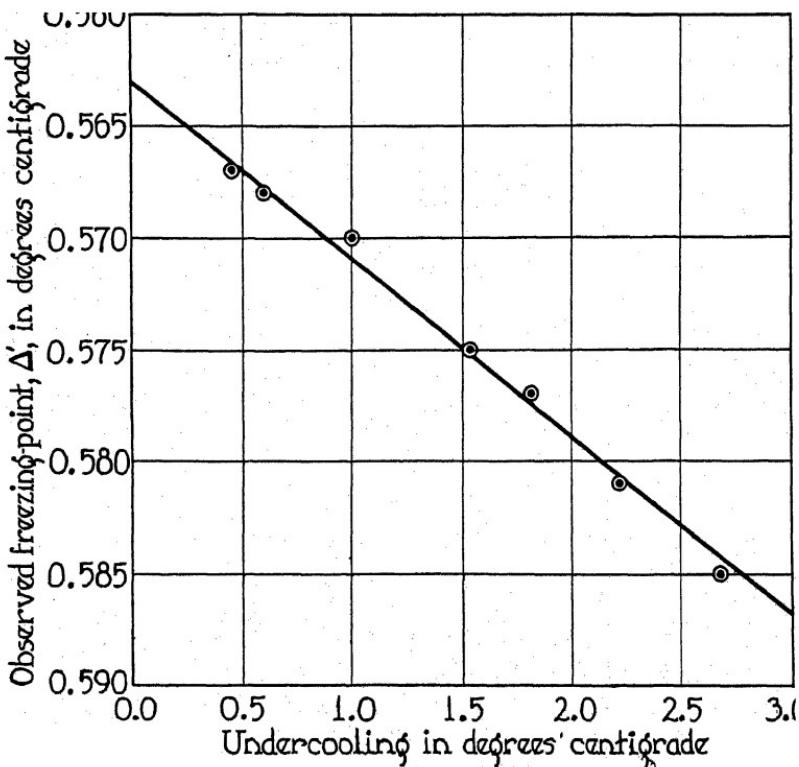
FIG. 1. Experiment 2. Observed freezing point depression and undercooling with a NaCl solution.

The problem of undercooling has been attacked experimentally. Materials of widely varying water content—potassium and sodium chloride solutions, serum, and corpuscles—have been used. In these experiments the observed freezing point, Δ', has been determined for a series of undercooling temperatures; *i.e.*, for undercoolings ranging from about 2.5–0.5°.

In order to initiate freezing at the desired point, it was necessary

to bring the fluid into contact with an ice crystal, to "inoculate." The method used was that of Beckmann, as described by Biltz (11), in which a rod of ice projecting from a glass tube is touched to the raised stirring rod through the side tube of the freezing point vessel.

The data have been treated graphically. The results of an experiment on a sodium chloride solution are plotted in Fig. 1. The respective undercooling, u, is recorded as an abscissa, and the observed freezing point, Δ', as an ordinate. The true freez-

TABLE I

Values of w

In Experiments 6, 7, 8, 12, 13, and 14 the blood was equilibrated with room air.

Experiment No.	w	Material	Experiment No.	w	Material
1	1.14	KCl solution	9	0.38	Corpuscles
2	0.90	NaCl "	10	0.33	"
			11	0.34	"
3	0.70	Serum	12	0.52	"
4	0.62	"	13	0.34	"
5	0.97	"	14	0.35	"
6	0.52	"			
7	0.70	"	Average...	0.38	
8	1.22	"			
Average....	0.79				

ing point, Δ, can be read off from Fig. 1 if we extrapolate. (The equation represents a hyperbola. However, in the range of values used, a practically straight line relationship can be shown to exist.) Δ represents the value of Δ' corresponding to zero undercooling, and in Fig. 1 is equal to $-0.563°$. By substituting this value of Δ and also a value of u and its corresponding Δ' (from the graph, when u equals $2°$, Δ' equals $-0.579°$) in the above equation of Collins and by solving, w can be determined. The value in this case is 0.90. In the same manner values of w have been calculated for all of the experiments. The results are given in Table I.

It should be noted that large errors are possible in determining w.

The experiments recorded above are the best of a number of experiments, and extreme care was required in order to obtain the close checks necessary in determining w. Relatively slight variations in the slope of the plotted line will cause large variations in this quantity.

w, which is the fraction by weight of water, should approximate unity for sodium and potassium chloride solutions. The results actually obtained were 0.90 and 1.14, which are in fairly good agreement with the expected value. The value of w, from published figures on water content, should be about 0.9 for serum and 0.6 for corpuscles (Abderhalden (16)). The averages found were 0.79 for serum and 0.38 for corpuscles. These results are difficult to interpret. It is possible to explain the discrepancies on the basis of "bound water," of water not active as a solvent. w theoretically represents the amount of "free" water, water which is able to act as a solvent. On this assumption the fraction of "bound water" would be 0.2 for corpuscles and 0.1 for serum. Since the existence of "bound water" is still a matter of controversy and since the experimental data on w vary considerably, correction for undercooling will be carried out in most cases with both theoretical (0.9 and 0.6) and experimental (0.8 and 0.4) values. In the following experiments undercooling has been limited by the above mentioned method of inoculation.

If, instead of assuming the specific heat to be unity, values of s of 0.95 for serum and 0.8 for corpuscles (Atzler and Richter (17)) are employed, the values obtained for w are smaller. The value for the corpuscles will obviously be influenced more than that for the serum. However, the final correction for undercooling is unaltered by the value used for s, since the change resulting in w due to inserting s is later neutralized. When the specific heats are considered, the new value of w will equal ws. If substitution is made in the correction equation, s will appear in both numerator and denominator and will thus cancel.

$$\Delta = \Delta' - \frac{su\ \Delta'}{80\ ws}$$

For this reason the specific heat has been assumed to be unity.

It should be noted that with this formula, in which the solid content is considered, that the correction is greater for corpuscles

than for serum; that is, a larger value is subtracted from the corpuscle reading than from the serum reading for the same amount of undercooling. Thus, if the investigators finding the serum to have the greater freezing point depression had applied the above correction, the differences obtained between the freezing points of serum and corpuscles would have been even greater than they found.

It should be noted that recently Johlin (18) and Stadie and Sunderman (19) have developed freezing point methods which involve very little undercooling. It would be interesting to repeat our work, employing these methods.

Evaporation during Centrifuging

It is very important to cork the tubes while centrifuging. The amount of evaporation which can occur is surprisingly great. For example, in two open tubes of 50 cc. volume filled with defibrinated beef blood and centrifuged for 45 minutes, the losses of weight were 1.46 and 1.45 gm. A 0.9 per cent sodium chloride solution with an original freezing point depression of 0.607° had, after 1 hour of centrifugation in open tubes, a Δ of 0.646°; after 2 hours, the Δ was 0.657°.

In the case of blood, since the evaporation presumably takes place at the surface, the concentration will probably be confined almost entirely to the serum. The result would be a greater freezing point depression for the serum than for the corpuscles. (It is probable that in uniform fluids convection or other currents might be set up to keep the composition more uniform.) A set of experiments was carried out to test this possibility. One sample of blood was centrifuged in stoppered tubes, while the other was centrifuged in open tubes. The stoppered tubes, after being filled completely to the top, were corked. The tubes had a volume of approximately 50 cc. The open tubes were about 11.5 cm. in length and 2.4 cm. in diameter. There was no constriction at the mouth as in the case of the closed tubes. With the open tubes the freezing point depression of the serum was 0.051° greater than that of the corpuscles; in the stoppered tubes the difference was only 0.001°. These averages are the result of six experiments done in duplicate. Thus evaporation during centrifugation results in a relative concentration of the serum. The results in open

tubes confirm the differences of the previous workers (except Kroenig and Fueth); while, if closed tubes are used, much smaller differences are obtained.

Comparison of Freezing Points of Serum and Corpuscles

A series of comparative determinations is presented in Table II in which the observed freezing points have been corrected as indicated above and in which stoppered centrifuge tubes were used. In the case of beef blood the difference between serum and corpuscles averaged 0.014°.[1] In the case of the dog the difference was smaller, namely 0.004°.[1] It will be seen that these average differences, although in the same direction (the serum having the greater freezing point depression), are much smaller than those obtained by previous workers (except Kroenig and Fueth). It should be pointed out again that the dog blood was arterial in character, while the beef blood was venous. The discrepancy between beef and dog blood might in part be explained by the more rapid escape of gas from the more venous beef corpuscles. A series of experiments has been done to test this point.

The Factor of the Blood Gases

In the preceding experiments no attempt was made to control the blood gases. One would expect corpuscles to lose their carbon dioxide more readily than the serum. Thus, if determinations were made under similar circumstances, the freezing point depression of the corpuscles might be less than that of the serum due to a greater loss of carbon dioxide from the corpuscles. Three types of experiments have been performed in an effort to eliminate this factor. (1) Freezing points have been determined on serum and corpuscles obtained from blood equilibrated with room air. (2) The blood has been handled and freezing points have been carried out in an atmosphere of alveolar air. (3) The procedure has been carried out under paraffin oil.

1. *Equilibration with Room Air*—If serum and corpuscles are obtained from blood equilibrated with room air, then there will obviously be little change in the carbon dioxide content either relatively or absolutely during manipulations such as occur in a

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[1] Correction for undercooling was made with experimental values of w.

freezing point determination. The results of this group of experiments are given in Table III. The equilibration was accomplished by rotation of the whole blood in a 2 liter bottle for 1 to 3 hours. A current of moist air (the air was in most cases bubbled through 0.8 per cent sodium chloride solution to moisten it) was either passed continuously over the blood, or the air in the bottle was changed frequently (five to nine times). After equilibration the blood was centrifuged in stoppered tubes, and the freezing points determined.

The results, when equilibrium with room air has been accomplished, approach each other more closely. The average difference is now 0.006^{o1} for beef blood and 0.002^{o1} for dog, the serum having the greater depression. This is approximately half the difference observed when no precautions as to escape of gas were taken.

2. Experiments Performed under Alveolar Air—An attempt has been made to keep the serum or corpuscles in contact with an atmosphere of alveolar air during the freezing point determinations, the blood being approximately in equilibrium with this air before centrifugation. Dog blood, obtained from the femoral artery and defibrinated in an open vessel, and beef blood, equilibrated with alveolar air, were used. Air from the last part of an expiration was considered to be alveolar air. The equilibration was accomplished by rotation for 1 to 3 hours in a 2 liter bottle. Air from the last part of every expiration, after being first bubbled through a 0.9 per cent sodium chloride solution, was passed into the bottle; or, as in two cases, five to six changes of alveolar air were made. All vessels and pipettes used in transferring and centrifuging the blood were previously filled with alveolar air. During the freezing point determination, air was blown with every breath into the freezing point tube. The results are given in Table IV. The average difference between serum and corpuscles is 0.012^{o1} for beef and 0.002^{o1} for dog blood, the serum in both cases being the more concentrated.

3. Experiments Performed under Paraffin Oil—In the following experiments all operations were carried out under paraffin oil in an effort to limit carbon dioxide loss. Centrifugation was carried out under oil. All transfers were made by means of pipettes into which some paraffin oil had previously been drawn. A layer of paraffin oil was thus always above the blood. The delivery was

TABLE II

Comparison of Freezing Points of Serum and Corpuscles. All values are given in °C.

"Brine" represents the temperature of the cooling brine.

Experiment No.	Serum					Corpuscles					$\Delta_s - \Delta_c$†
	Δ'	u	Δ_t^*	Δ_e^*	Brine	Δ'	u	Δ_t^*	Δ_e^*	Brine	
						Beef blood					
21	−0.630	0.18	−0.628	−0.628		−0.587	0.26	−0.584	−0.582		−0.046
	−0.633	0.27	−0.631	−0.630		−0.583		−0.583	−0.583		
22	−0.598	0.52	−0.594	−0.593		−0.585	0.28	−0.582	−0.580		−0.010
	−0.593	0.73	−0.587	−0.586		−0.580		−0.580	−0.580		
23	−0.595	0.62	−0.590	−0.589		−0.600	0.31	−0.596	−0.594		−0.004
	−0.597	0.30	−0.595	−0.594		−0.590	0.45	−0.584	−0.582		
24	−0.632	0.41	−0.628	−0.628		−0.652	0.30	−0.648	−0.646		+0.005
	−0.635	0.28	−0.632	−0.632		−0.625	0.05	−0.624	−0.624		
25	−0.587	0.22	−0.585	−0.585		−0.567	0.30	−0.563	−0.562		−0.030
	−0.595	0.18	−0.594	−0.593		−0.559	0.15	−0.557	−0.556		
26	−0.590	0.78	−0.584	−0.583		−0.577	0.76	−0.568	−0.563		−0.021
	−0.594	1.17	−0.584	−0.583		−0.579	0.95	−0.568	−0.562		
	−0.607	2.61	−0.585	−0.582		−0.580	0.98	−0.568	−0.562		
27	−0.566	0.63	−0.561	−0.560	−3.5	−0.557	0.69	−0.549	−0.545	−3.3	−0.016
	−0.567	0.63	−0.562	−0.561	−3.5	−0.557	0.69	−0.549	−0.545	−3.3	
28	−0.580	0.65	−0.575	−0.574	−4.0	−0.585	0.65	−0.577	−0.573	−3.5	−0.003
	−0.580	0.69	−0.574	−0.574	−4.0	−0.584	0.81	−0.574	−0.569	−3.5	
29	−0.583	0.73	−0.577	−0.576	−4.0	−0.590	0.82	−0.580	−0.575	−4.0	+0.001
	−0.583	0.71	−0.577	−0.577	−4.0	−0.592	0.67	−0.584	−0.580	−3.5	
30	−0.580	0.84	−0.573	−0.572	−4.3	−0.587	0.59	−0.580	−0.576	−3.7	+0.005
	−0.578	0.84	−0.571	−0.570	−4.3	−0.589	0.76	−0.580	−0.575	−3.7	
31	−0.600	0.80	−0.593	−0.593	−4.5	−0.599	0.71	−0.590	−0.586	−4.0	

No.											
32	−0.602	0.82	−0.595	−0.594	−4.1	−0.601	0.80	−0.591	−0.586	−4.0	−0.008
	−0.545	0.75	−0.539	−0.539	−3.3	−0.543	0.78	−0.534	−0.530	−3.0	
33	−0.546	0.67	−0.541	−0.540	−3.3	−0.542	0.62	−0.535	−0.531	−5.0	−0.009
	−0.600	0.65	−0.595	−0.594	−4.3	−0.570	0.82	−0.560	−0.555	−4.0	
34	−0.600	0.87	−0.593	−0.592	−4.0	−0.567	0.68	−0.559	−0.555	−4.0	−0.038
	−0.590	0.59	−0.585	−0.585	−4.0	−0.570	0.63	−0.563	−0.559	−3.2	−0.026
	−0.590	0.62	−0.585	−0.584	−4.0	−0.570	0.68	−0.562	−0.558	−3.2	
Average											−0.0143
Dog blood											
35	−0.610	0.26	−0.608	−0.608		−0.590	0.19	−0.588	−0.586		−0.019
	−0.608	0.37	−0.605	−0.604		−0.588	0.01	−0.588	−0.588		
36	−0.590	0.58	−0.585	−0.585		−0.595	0.38	−0.595	−0.595		+0.003
	−0.590	0.27	−0.588	−0.588		−0.592		−0.587	−0.585		
37	−0.607	0.28	−0.605	−0.604		−0.614	0.33	−0.610	−0.608		+0.007
	−0.607	0.43	−0.603	−0.603		−0.620	0.29	−0.616	−0.614		
38	−0.630	0.35	−0.627	−0.627		−0.613	0.31	−0.609	−0.607		−0.017
	−0.628	0.21	−0.626	−0.626		−0.615	0.08	−0.614	−0.613		
39	−0.620	0.03	−0.620	−0.620		−0.622	0.38	−0.617	−0.615		−0.006
	−0.622	0.26	−0.620	−0.619		−0.612		−0.612	−0.612		
40	−0.612	0.16	−0.611	−0.610		−0.632	0.34	−0.627	−0.625		+0.012
	−0.612	0.21	−0.610	−0.610		−0.620	0.03	−0.620	−0.619		
41	−0.612	0.29	−0.610	−0.609		−0.625	0.38	−0.620	−0.618		+0.004
	−0.607	0.15	−0.606	−0.606		−0.612	0.46	−0.606	−0.603		
	−0.609	0.41	−0.606	−0.605							

TABLE II—*Concluded*

Dog blood—*Concluded*

Experiment No.	Serum					Corpuscles					$\Delta_s - \Delta_c$
	Δ'	u	Δ_l^*	Δ_e^*	Brine	Δ'	u	Δ_l^*	Δ_e^*	Brine	
42	-0.622	0.33	-0.619	-0.619		-0.645	0.50	-0.638	-0.635		+0.016
	-0.622	0.13	-0.621	-0.621		-0.639	0.27	-0.635	-0.634		
	-0.622	0.37	-0.619	-0.618							
43	-0.597	0.42	-0.594	-0.593		-0.572	0.51	-0.566	-0.563		-0.026
	-0.595	0.25	-0.593	-0.593		-0.577	0.39	-0.572	-0.570		
44	-0.595	0.56	-0.590	-0.590		-0.597	0.42	-0.592	-0.589		-0.008
	-0.605	0.16	-0.604	-0.603		-0.595	0.34	-0.591	-0.589		
45	-0.622	0.46	-0.618	-0.618		-0.587	0.17	-0.585	-0.584		-0.035
	-0.612	0.12	-0.611	-0.611		-0.587	0.64	-0.579	-0.575		
46	-0.716	0.41	-0.712	-0.711		-0.737	0.45	-0.730	-0.727		+0.017
	-0.715	0.65	-0.709	-0.708		-0.737	0.42	-0.731	-0.727		
47	-0.580	0.63	-0.575	-0.574	-3.0	-0.593	0.41	-0.588	-0.585	-3.0	+0.010
	-0.582	0.63	-0.577	-0.576	-3.0	-0.592	0.40	-0.587	-0.585	-3.0	
48	-0.560	0.64	-0.555	-0.554	-3.0	-0.540	0.67	-0.532	-0.529	-3.2	-0.026
	-0.558	0.63	-0.553	-0.553	-2.8	-0.538	0.69	-0.530	-0.526	-3.2	
49	-0.582	0.86	-0.575	-0.574	-2.5	-0.590	0.96	-0.578	-0.572	-4.0	-0.003
	-0.585	0.32	-0.582	-0.582	-2.5	-0.585	0.43	-0.580	-0.577	-4.0	
50	-0.577	0.86	-0.570	-0.569	-4.0	-0.581	0.61	-0.574	-0.570	-3.5	-0.001
	-0.577	0.61	-0.572	-0.572	-3.8	-0.585	0.86	-0.575	-0.569	-3.3	
51	-0.574	0.77	-0.568	-0.567	-4.5	-0.583	0.65	-0.575	-0.571	-4.2	+0.002
	-0.575	0.58	-0.570	-0.570	-4.5	-0.583	0.70	-0.574	-0.570	-4.2	

52	−0.570	0.71	−0.564	−0.564	−4.0	−0.580	0.84	−0.570	−0.565	−3.8	+0.001
	−0.570	0.66	−0.565	−0.564	−4.0	−0.580	0.84	−0.570	−0.565	−3.8	
53	−0.567	0.67	−0.562	−0.561	−3.5	−0.576	0.79	−0.567	−0.562	−4.3	+0.002
	−0.569	0.90	−0.562	−0.561	−4.5	−0.577	0.78	−0.568	−0.563	−4.3	
54	−0.570	0.66	−0.565	−0.564	−3.7	−0.570	0.82	−0.560	−0.555	−3.3	−0.007
	−0.570	0.74	−0.564	−0.563	−3.7	−0.570	0.67	−0.562	−0.558	−3.3	
55	−0.585	0.80	−0.578	−0.578	−3.0	−0.592	0.75	−0.583	−0.578	−3.0	−0.001
	−0.585	0.55	−0.580	−0.580	−3.0	−0.587	0.51	−0.581	−0.578	−3.0	
Average											−0.0036

* $\Delta_t = \Delta$ obtained by using theoretical values of w.　$\Delta_e = \Delta$ obtained by using experimental values of w.
† These Δ values were obtained by using experimental values of w.

TABLE III

Blood in Equilibrium with Room Air

"Brine" represents the temperature of the cooling brine. All values are given in °C.

Experiment No.	Serum Δ'	u	Δ_t	Δ_e	Brine	Corpuscles Δ'	u	Δ_t	Δ_e	Brine	$\Delta_s - \Delta_c^{*}$
					Beef blood						
56	−0.576	0.65	−0.571	−0.570	−3.2	−0.580	0.81	−0.570	−0.565	−4.5	−0.005
	−0.577	0.58	−0.572	−0.572	−3.2	−0.577	0.62	−0.570	−0.566	−4.2	
57	−0.526	0.72	−0.521	−0.520	−3.8	−0.530	0.67	−0.523	−0.519	−3.5	−0.001
	−0.529	0.79	−0.523	−0.522	−3.5	−0.530	0.62	−0.523	−0.520	−3.5	
58	−0.544	0.58	−0.540	−0.539	−4.5	−0.540	0.63	−0.533	−0.529	−4.0	−0.010
	−0.551	0.62	−0.546	−0.546	−4.2	−0.544	0.98	−0.533	−0.527	−3.2	
	−0.537	0.64	−0.532	−0.532	−3.0	−0.548	1.27	−0.534	−0.526	−3.7	
	−0.540	0.66	−0.535	−0.534	−3.0	−0.552	1.54	−0.534	−0.525	−3.5	
	−0.542	0.83	−0.536	−0.535	−4.0						
	−0.542	0.88	−0.535	−0.535	−3.5						
	−0.543	0.98	−0.536	−0.535	−3.5						
	−0.550	1.17	−0.541	−0.540	−3.5						
59	−0.551	0.60	−0.546	−0.546	−3.3	−0.540	0.68	−0.532	−0.529	−3.5	−0.008
	−0.542	0.68	−0.537	−0.536	−3.5	−0.542	0.71	−0.534	−0.530	−3.3	
	−0.542	0.72	−0.537	−0.536	−3.0	−0.542	0.73	−0.534	−0.530	−3.1	
	−0.541	0.73	−0.536	−0.535	−3.0						
60	−0.562	0.64	−0.557	−0.556	−3.8	−0.562	0.61	−0.555	−0.551	−3.9	−0.006
	−0.562	0.93	−0.555	−0.554	−3.7	−0.563	1.64	−0.563	−0.553	−3.9	
	−0.570	1.30	−0.560	−0.558	−3.8	−0.573	1.24	−0.558	−0.551	−3.9	
	−0.566	0.95	−0.559	−0.558	−3.6	−0.565	0.86	−0.555	−0.550	−3.9	

.61	−0.564	0.96	−0.556	−0.556	−3.9	−0.570	1.05	−0.558	−0.551	−3.9	
	−0.568	1.05	−0.560	−0.559	−3.8	−0.574	1.17	−0.560	−0.553	−3.8	−0.005
	−0.570	1.47	−0.558	−0.557	−3.9	−0.564	0.67	−0.556	−0.552	−3.8	
62	−0.553	0.93	−0.546	−0.545	−3.7	−0.563	1.43	−0.546	−0.538	−4.1	
	−0.551	0.62	−0.546	−0.546	−3.7	−0.561	1.08	−0.548	−0.542	−3.9	
	−0.551	0.63	−0.546	−0.546	−3.9	−0.550	0.82	−0.541	−0.536	−3.9	
	−0.553	1.11	−0.544	−0.543	−3.8	−0.549	0.62	−0.542	−0.538	−3.9	
	−0.552	0.59	−0.547	−0.547	−3.8	−0.550	0.54	−0.544	−0.541	−3.9	
	−0.554	1.09	−0.546	−0.545	−3.7	−0.556	0.71	−0.548	−0.544	−4.0	−0.005
	−0.555	0.84	−0.549	−0.548	−3.9	−0.571	1.47	−0.554	−0.545	−4.0	
	−0.553	0.84	−0.547	−0.546	−3.9						
	−0.556	1.40	−0.545	−0.544	−3.9						
Average											−0.0057

Dog blood

63	−0.558	0.84	−0.551	−0.551	−4.8	−0.560	0.74	−0.551	−0.547	−4.0	−0.005
	−0.558	0.82	−0.552	−0.551	−4.6	−0.558	0.76	−0.549	−0.545	−4.0	
64	−0.585	0.71	−0.579	−0.579	−4.8	−0.594	0.56	−0.587	−0.584	−4.0	+0.001
	−0.584	0.69	−0.578	−0.578	−4.6	−0.590	0.76	−0.581	−0.576	−4.0	
Average											−0.002

made into oil, which was placed at the bottom of the vessel into which delivery was to be made.

The vessels used for the freezing point determinations were of a different type than those previously employed. The blood was contained in a short tube, 6 cm. in length and 2 cm. in diameter. Through a rubber stopper, inserted into the top of this vessel, there passed the following: the stem of the Beckmann thermometer; a long, narrow glass tube, 10 cm. long and 1.5 mm. in diameter, through which passed the stirring rod surrounded with vaseline; a shorter glass tube of 4 mm. bore left open for overflow; and a solid glass rod. The vessel was first completely filled with serum or corpuscles (under oil, of course). The stopper, with its tubes and thermometer, was then pushed into the vessel, displacing the oil on top and also some of the top layers of blood. Oil was placed on the surface of the blood which had risen into the short open tube. When the stirrer moved up and down, the level changed in the short open tube due to the volume change with the varying amount of the stirrer actually in the freezing point vessel. (No fluid could enter the fine glass tube containing the stirring rod because of the vaseline.) Inoculation was accomplished through the fourth opening in the rubber stopper. In the first part of the determination this hole was plugged with the solid glass rod. When it was desired to bring about freezing, the solid rod was removed, and there was quickly inserted a glass tube of the same size with water frozen in its end. During the determination, the whole apparatus was placed in a large test-tube, which was immersed in the cooling mixture.

Preliminary control experiments were performed to check the above technique. It was thought that the modifications might in some way invalidate the values obtained for the freezing point. For example, the paraffin oil might contain soluble impurities, or it might dissolve some of the constituents of the serum or corpuscles. Freezing points on identical materials were determined by the ordinary method and also by the paraffin oil technique, and the results compared. Such determinations were made on distilled water, beef serum, and beef corpuscles. Since the serum and corpuscles were in equilibrium with room air, the blood gas factor was eliminated. The results showed practically exact agreement between the values obtained by the two techniques. Hence, the

TABLE IV

Experiments Performed under Alveolar Air

...ure of the cooling brine. All values are given in °C.

No.						...od					
65	−0.572	0.64	−0.567	−0.566	−4.2	−0.572	0.59	−0.565	−0.561	−4.2	
66	−0.572	0.82	−0.565	−0.565	−4.2	−0.577	0.68	−0.569	−0.565	−4.0	−0.003
	−0.600	0.56	−0.595	−0.595	−4.7	−0.603	0.76	−0.593	−0.589	−4.0	
67	−0.602	0.66	−0.596	−0.596	−4.7	−0.598	0.69	−0.589	−0.585	−4.2	−0.009
	−0.610	0.64	−0.605	−0.604	−4.3	−0.604	0.74	−0.595	−0.590	−3.5	
68	−0.610	0.60	−0.605	−0.604	−4.2	−0.607	0.48	−0.601	−0.598	−4.2	−0.010
	−0.634	0.77	−0.627	−0.626	−3.5	−0.636	0.47	−0.630	−0.627	−3.0	
69	−0.636	0.86	−0.628	−0.627	−3.5	−0.633	0.82	−0.622	−0.617	−3.0	−0.005
	−0.600	0.56	−0.595	−0.595	−4.0	−0.570	0.54	−0.564	−0.560	−3.5	
	−0.599	0.76	−0.593	−0.592	−3.6	−0.570	0.68	−0.562	−0.558	−3.0	−0.035
	−0.600	0.66	−0.594	−0.594	−3.6						
Average	....										−0.0124

Dog blood

No.											
70	−0.570	0.82	−0.564	−0.563	−4.0	−0.572	0.61	−0.565	−0.561	−3.5	−0.004
	−0.570	0.61	−0.565	−0.565	−4.0	−0.570	0.68	−0.562	−0.558	−3.3	
71	−0.557	0.65	−0.552	−0.551	−3.5	−0.560	0.55	−0.554	−0.550	−3.3	−0.000
	−0.559	0.58	−0.554	−0.554	−3.5	−0.570	0.84	−0.560	−0.555	−3.0	
Average	....										−0.002

Bi

Experiment No.	Serum					Corpuscles					$\Delta_s - \Delta_c{}^*$
	Δ'	u	Δ_t	Δ_e	Brine	Δ'	u	Δ_t	Δ_e	Brine	
					Beef blood						
78	−0.552	1.04	−0.544	−0.543	−5.0	−0.546	0.55	−0.540	−0.537	−5.0	−0.010
	−0.549	0.51	−0.545	−0.545	−4.5	−0.554	1.06	−0.542	−0.536	−4.5	
	−0.553	1.04	−0.545	−0.544	−4.2	−0.552	1.05	−0.540	−0.534	−4.0	
	−0.554	0.24	−0.552	−0.552	−4.0						
	−0.552	0.99	−0.544	−0.543	−4.0						
	−0.550	0.49	−0.546	−0.546	−4.0						
79	−0.586	0.52	−0.582	−0.581	−3.3	−0.584	0.59	−0.577	−0.573	−4.0	−0.008
	−0.583	0.59	−0.578	−0.578	−3.0	−0.581	0.63	−0.573	−0.570	−3.8	
80	−0.562	0.53	−0.558	−0.557	−4.3	−0.558	0.58	−0.551	−0.548	−5.0	−0.007
	−0.560	0.58	−0.555	−0.555	−4.0	−0.560	0.56	−0.553	−0.550	−4.0	
Average	....										−0.0083
					Dog blood						
81	−0.583†	0.59	−0.578	−0.578	−3.0	−0.575†	0.40	−0.570	−0.568	−4.0	−0.011
	−0.581†	0.73	−0.575	−0.574	−3.0	−0.580†	1.04	−0.567	−0.561	−4.0	
82	−0.562†	0.57	−0.557	−0.557	−3.0	−0.560†	0.65	−0.552	−0.549	−3.3	−0.008
	−0.563†	0.65	−0.558	−0.557	−3.0	−0.560†	0.60	−0.553	−0.549	−3.0	
	−0.568‡	0.60	−0.563	−0.563	−3.3	−0.570‡	0.64	−0.562	−0.559	−3.5	−0.004
	−0.570‡	0.59	−0.565	−0.565	−3.0	−0.570‡	0.53	−0.564	−0.561	−3.0	
Average	....										−0.0077

method is justified—the modifications do not introduce significant errors.

The effectiveness of the paraffin oil method in preventing changes in the blood gases was directly determined by gas analysis. The oxygen and carbon dioxide contents of the blood were determined before and after a paraffin oil freezing point determination by means of a Van Slyke manometric blood gas apparatus (Van Slyke and Neill (20), Van Slyke (21)). The changes were small, being less than 1 volume per cent for carbon dioxide and still smaller for oxygen. Such a difference could hardly be expected to cause a measurable difference in the freezing point. Consider the fact that the difference in the freezing point of venous and arterial blood is probably not more than 0.01° (Nolf (22),[2] while the difference in carbon dioxide between venous and arterial blood is about 6 volumes per cent in the dog (see Starling (23)).

The experimental data on the freezing points of serum and corpuscles, determined by the above method, are given in Table V. The average difference is 0.008°[1] for both beef and dog blood, the serum having the greater Δ.

The mean differences in freezing points ($\Delta_s - \Delta_c$) for all the experiments in which the blood gases were controlled are presented below. A minus sign indicates that the serum is more concentrated than the corpuscles, and a plus sign indicates the opposite.

| Kind of blood | Differences | | Method of CO_2 control |
	w_t*	w_e†	
	°C.	°C.	
Beef............................	−0.001	−0.006	Equilibration with room
Dog............................	+0.002	−0.002	air
Beef............................	−0.009	−0.012	Procedure carried out
Dog............................	+0.002	−0.002	under alveolar air
Beef............................	−0.005	−0.008	Paraffin oil technique
Dog............................	−0.005	−0.008	

* Correction for undercooling was made with theoretical values of w.
† Correction for undercooling was made with experimental values of w.

[2] Using the paraffin oil technique, we have found differences of not more than 0.01°. See Table V.

If all of the experiments in which the blood gases were controlled are averaged, the following mean differences are obtained.

Kind of blood	Differences	
	W_t* °C.	W_e† °C.
Beef.....................................	−0.005	−0.009
Dog.....................................	−0.001	−0.004

For the sake of comparison, the average results of the uncontrolled carbon dioxide experiments are given.

Kind of blood	Differences	
	W_t* °C.	W_e† °C.
Beef.....................................	−0.012	−0.014
Dog.....................................	−0.001	−0.004

Comparing the last two tabulations, one sees in the case of the dog blood, which is arterial, that the average differences between serum and corpuscles are the same in the controlled and in the uncontrolled carbon dioxide experiments. In the beef blood, which is very venous, carbon dioxide control seems to exert some effect, for the average differences are less when carbon dioxide loss is controlled. However, the effect of controlling the gaseous loss is, if anything, small. A statistical treatment of the data at hand fails to show any significance.

Temperature of Centrifuging

Another possible cause for error lies in the fact that the separation of serum from corpuscles by centrifugation is carried out at room temperature. Cooling probably causes unequal changes in the osmolar concentration of cells and serum. Thus the data of Jacobs and Parpart (24) indicate that the red blood cells increase in volume when the temperature is lowered due to a decreased base-binding power of hemoglobin. This possibility for error may be eliminated by centrifugation at the freezing point. If unequal changes have taken place, there has been opportunity for water exchange to occur between cells and serum and thus to equalize the osmotic pressures.

Experiments, designed to test the effect of the temperature of

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* Correction for undercooling was made with theoretical values of w.
† Correction for undercooling was made with experimental values of w.

<div align="center">TABLE VI</div>

Freezing Points of Whole Blood

Theoretical values of w have been used in the correction for undercooling. For whole blood a value of w equal to 0.8 was used. In the case of serum and corpuscles, only Δ has been recorded since the other values are given in previous tables. "Brine" represents temperature of cooling brine.

	Experiment No.	Type of blood	Serum	Whole blood				Corpuscles
				Δ'	u	Δ	Brine	
			°C.	°C.	°C.	°C.	°C.	°C.
No control of blood gases	32	Beef	−0.539	−0.542	0.68	−0.536	−3.8	−0.534
			−0.541	−0.545	0.77	−0.538	−3.8	−0.535
	33	"	−0.595	−0.583	0.64	−0.577	−4.3	−0.560
			−0.593	−0.583	0.55	−0.578	−3.5	−0.559
	34	"	−0.585	−0.572	0.56	−0.567	−4.5	−0.563
			−0.585	−0.572	0.74	−0.565	−4.5	−0.562
	55	Dog	−0.578	−0.572	0.85	−0.564	−5.0	−0.583
			−0.580	−0.570	1.00	−0.561	−5.0	−0.581
Equilibration with room air	56	Beef	−0.571	−0.576	0.78	−0.569	−3.6	−0.570
			−0.572	−0.577	0.58	−0.572	−3.4	−0.570
	57	"	−0.521	−0.526	0.85	−0.519	−4.3	−0.523
			−0.523	−0.523	0.72	−0.517	−4.2	−0.523
	58	Dog	−0.551	−0.557	0.76	−0.550	−4.5	−0.551
			−0.552	−0.556	1.04	−0.547	−4.3	−0.549
				−0.557	0.94	−0.549	−4.0	
	59	"	−0.579	−0.585	0.67	−0.579	−5.0	−0.587
			−0.578	−0.584	0.67	−0.578	−4.0	−0.581
				−0.586	0.81	−0.579	−3.5	
Alveolar air	65	Beef	−0.567	−0.576	0.78	−0.569	−4.5	−0.565
			−0.565	−0.572	0.86	−0.564	−4.2	−0.569
	66	"	−0.595	−0.597	0.66	−0.591	−5.0	−0.593
			−0.596	−0.597	0.66	−0.591	−5.0	−0.589
	67	"	−0.605	−0.600	0.56	−0.595	−4.2	−0.595
			−0.605	−0.602	0.58	−0.597	−4.2	−0.601
	68	"	−0.627	−0.626	0.88	−0.617	−4.0	−0.630
			−0.628	−0.626	0.98	−0.616	−4.0	−0.622
	69	"	−0.595	−0.582	0.73	−0.575	−3.5	−0.564
			−0.593	−0.582	0.56	−0.577	−3.5	−0.562
			−0.594					
	70	Dog	−0.564	−0.570	0.68	−0.564	−4.0	−0.565
			−0.565	−0.570	0.82	−0.563	−4.0	−0.562
	71	"	−0.552	−0.550	0.60	−0.545	−5.0	−0.554
			−0.554	−0.556	0.70	−0.550	−4.5	−0.560
				−0.558	0.55	−0.553	−4.5	

TABLE VI—*Concluded*

	Experiment No.	Type of blood	Serum	Whole blood				Corpuscles
				Δ′	u	Δ	Brine	
			°C.	°C.	°C.	°C.	°C.	°C.
Paraffin oil method	78	Beef	−0.544	−0.550	1.03	−0.541	−5.0	−0.540
			−0.545	−0.542	1.03	−0.533	−4.5	−0.542
			−0.545	−0.534	0.56	−0.529	−4.05	−0.540
			−0.552					
			−0.544					
			−0.546					
	79	"	−0.582	−0.568	0.70	−0.562	−5.0	−0.577
			−0.578	−0.574	0.66	−0.568	−4.3	−0.573
				−0.570	0.58	−0.565	−4.0	
	80	"	−0.558	−0.556	0.56	−0.551	−3.7	−0.551
			−0.555	−0.558	0.57	−0.553	−5.0	−0.553
				−0.555	0.53	−0.550	−4.7	
	81	Dog	−0.578	−0.573	0.73	−0.566	−3.0	−0.570
			−0.575	−0.572	0.74	−0.565	−3.5	−0.567
	82	"	−0.557	−0.548	0.64	−0.543	−4.0	−0.552
			−0.558	−0.548	0.61	−0.543	−4.0	−0.553
			−0.563*	−0.555*	0.55	−0.550	−4.0	−0.562*
			−0.565*	−0.553*	0.57	−0.548	−3.5	−0.564*

* Venous blood; all other dog blood was arterial.

centrifugation, were performed. Defibrinated beef blood, equili-brated with room air, was divided into two parts. After being kept for 3 to 6 hours either at room temperature (about 20°) or at 0°, the samples were centrifuged at their respective tempera-tures. The centrifugation at 0° was performed out of doors during the winter season, and the tubes containing the blood were packed in ice in large centrifuge cups. A longer period of centrifugation was necessary at 0° since the corpuscles sediment much more slowly when the blood is cold. Practically identical freezing points and differences were obtained regardless of the temperature of centrifugation. As far as freezing points are concerned, it makes little difference at what temperature the cells and serum are separated.

Whole Blood

How does the freezing point of whole blood compare with that of serum and corpuscles? The literature on this question is con-

tradictory. Collip (5) finds whole blood to be intermediate between serum and corpuscles. According to Hamburger (25) (and also Stewart (2)) the whole blood and the serum have the same freezing points, while Japelli (26) and Nolf (22) find the latter to have a somewhat greater freezing point depression than the former.

It is interesting that Grollman (27), in vapor pressure measurements, obtained equal values for whole blood and plasma. He used a dynamic method at body temperature. The gas passed over the blood had the composition of alveolar air; consequently there were no errors caused by escape of carbon dioxide. Heparinized dog blood, obtained from unanesthetized animals, was employed.

In our experiments (Table VI) the freezing point of the whole blood corresponded fairly well with that of serum and of corpuscles, seemingly following the corpuscles more closely.

DISCUSSION

Are the discrepancies between cells and serum obtained in the present researches significant? Does the corpuscle actually have a smaller freezing point depression than the serum? It must be remembered that the differences recorded are small. (The reader is referred to the tabulations at the end of the section on blood gases.) For example, in the carbon dioxide-controlled experiments, if experimental values of w are used in the correction for undercooling, the average differences are 0.009° and 0.004° for beef and dog blood respectively; when theoretical values of w are employed, the variations are only 0.005° and 0.001°. (In the above the serum always has the greater freezing point depression.) The chances for experimental error seem to us to render these differences of doubtful significance. For example, one possibility for error lies in the high viscosity of cells as compared with serum. This high viscosity will result in more friction and hence more heat on stirring. The freezing point depression measured for the corpuscles may thus be erroneously small. Another chance for error lies in the poorer stirring of the corpuscles due to their high viscosity. Because of the possible experimental error and because of the small differences obtained, we cannot conclude that these experiments demonstrate any significant difference between the freezing points of serum and corpuscles.

SUMMARY AND CONCLUSION

The relatively large differences found by many previous investigators between the freezing points of serum and the corresponding corpuscles, may be explained on the basis of certain errors in the procedure.

The factors of error in such freezing point determinations are:

Evaporation in Centrifugation—It is essential to stopper tubes containing blood during centrifugation. If this precaution is not taken, a relative concentration of the serum due to evaporation occurs; and erroneously high differences between serum and corpuscles, comparable to those of previous investigators, are obtained.

Undercooling—In materials of relatively low water content, such as corpuscles, it is especially necessary to consider solid content in the correction for undercooling. A formula, which accounts for the solid content, has been developed.

Loss of Carbon Dioxide—The relative error resulting from carbon dioxide loss is comparatively small.

Temperature of Centrifugation—Little error results from centrifuging at room temperature and then performing the freezing point determinations at about 0°.

If proper correction is made for undercooling and if the blood is centrifuged in stoppered tubes, the differences obtained between the freezing points of serum and corpuscles are small, and probably within experimental error.

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IRON RETENTION BY WOMEN DURING PREGNANCY*

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The high iron content of new born mammals has long been
known. It was Bunge (1) who doubted whether it would be
possible during the relatively short period of pregnancy for the
mother to assimilate enough iron from her food to provide the
new-born with the quantity which it possesses at birth. He
suggested that the female organism, beginning even at puberty,
puts aside reserves of iron for this future need of reproduction, and
that the withdrawal of this iron from circulation is an explanation
of the frequent chlorosis in adolescent girls.

A study of the retention of iron during pregnancy in the human
organism might be expected to throw some light on this question
of assimilation raised by Bunge. Besides showing how much iron
is retained during gestation, it would indicate the effect of con-
stituents of the diet and other factors on iron metabolism. No
such information seems to be available for estimating the iron
requirement of pregnant women. With the hope of contributing
to the beginning of the accumulation of these facts, data for
twenty-three iron balances on nine women at different stages of
pregnancy are presented in this paper.

Many of the investigations on iron metabolism with human subjects antedate our present knowledge of nutrition as well as the use of micro-chemical methods of analysis. Those previous to 1907 were reviewed by Sherman (2). A recent excellent discussion of the literature pertaining to the rôle of iron in nutrition is included in the nutrition report (3) of the Committee on Growth and Development of the Child by the Medical Section of the White House Conference. This survey summarizes the published data on the iron content of the body and various parts thereof, that from metabolism studies both with adults and with children, and that on the present status of our information relating to the absorption and excretion of iron and the various factors influencing its metabolism. Another study with pre-school children (4) has been published lately. So far as the writer is aware, no metabolism studies with women subjects during pregnancy have been reported.

The Committee's reviewers (3) estimated the fetal demands by calculating from the figures of analyses of human fetuses reported by Hugounenq (5), by Brubacher (6), and by Camerer and Söldner (7). They show that the average daily transfer of iron from mother to fetus amounted to about 0.4 mg. during the first two-thirds of pregnancy but that during the last third of the period the accretion was 10 times greater, or about 4.7 mg. of iron daily. A total iron content of 375 mg. in the mature human fetus was accepted as a fair average from the various analyses reported.

The maternal organism has additional needs for iron in hypertrophied muscles and other tissues, and especially in the system of placental circulation which must be developed early in the period of gestation. According to Vierordt's tables (8), 5 mg. per cent by weight may be allowed for the iron in muscles and 54 mg. per cent for that in the blood of the human adult.

Some recent work with laboratory animals on diets more nearly adequate than those used by early investigators tend to confirm the findings of the latter respecting the iron content of the young. Smythe and Miller (9) found that the absolute amounts of iron in the bodies of young rats remained fairly constant through the nursing period, but that the percentage iron content decreased to the lowest point by weaning time. This fact, as well as that of the low iron content of milk, emphasizes the task of the maternal organism in providing the fetus with reserve supplies of iron sufficient to last the growing offspring until the end of the nursing period.

Smythe and Miller also showed that the iron content of adult rats, just after giving birth to young, averaged some 20 per cent lower than that of females which had not reproduced. The depleted reserves were apparently back to normal by weaning time.

Kojima (10) made similar observations on the amount of iron in different organs of the pregnant rat. Only the uterus was higher in iron than was that of non-pregnant females.

The subjects of this experiment were those Chicago women for whom other metabolism studies have been reported (11). The details of the methods of collection and variations in the food habits of subjects have been discussed in previous papers (12). Only points pertinent to iron studies will be reiterated here.

The feces were collected and dried in Pyrex glass containers and the food composites in glazed porcelain evaporating dishes. During the drying process, which was carried out on a copper water bath, the samples were kept covered with paper toweling. Only iron-free chemicals were used for preservation of urine and feces. Porcelain or nickel spatulas and a silver spoon were used in handling the material both before and after drying. The dried food and feces were ground in a porcelain mortar and stored in bottles, the tops of which were tightly covered with cloth and brown paper, until analyzed.

The difficulties associated with the determination of iron in biological materials are responsible in a large measure for the paucity of data on iron metabolism. Much has been written recently on methods both old and new for analysis of such small quantities of iron as occur in food and excreta (13–17). Only enough description of the methods used in this series is given to indicate the probable accuracy of the data presented.

Iron was determined by the thiocyanate method with amyl alcohol. For most of the samples the alkali hydrolysis was employed. The details, with minor modifications, were those described by Ascham (16). The material was dry-ashed in platinum, first over a flame from a brass burner and on a glazed tile support, then in an electric muffle furnace at a temperature just below red heat. The ash was dissolved in HCl, made slightly alkaline with NaOH, and boiled 1 hour to convert the pyrophosphates. The solution was then made acid with sulfuric acid and an aliquot, with acidity adjusted and KCNS added, was shaken with amyl alcohol and the color compared to a standard made up simultaneously with exactly the same reagents.

An acid hydrolysis, similar to that described by Stugart (13), but with less acid in the standard, was used for all urine deter-

minations. This procedure gave results identical with those from the alkali hydrolysis, but avoided the supersaturated solutions frequently occurring as a result of the salts formed by the successive neutralizations together with the original salts in the urine.

The difficulties with alkali hydrolysis cited by Stugart were not encountered in this series. The solutions were made barely alkaline and boiled gently so that at no time after the hydrolysis was etching of the Pyrex beaker observed. The results were checked exactly or with slightly higher figures from the acid hydrolysis on a series of both food and feces. Furthermore, one lot of sodium hydroxide crystals in which iron could not be detected in the amounts used for these determinations was obtained and used throughout these analyses. Attempts to purify iron-containing alkalies, even by allowing solutions to stand in Pyrex for 2 years, failed completely. Obtaining an iron-free thiocyanate gave much difficulty, one high grade product being found to contain nearly 10 times as much iron as the manufacturer's label indicated.

The method with alkali hydrolysis and the sulfuric acid medium yields a purer quality of color than does the acid hydrolysis, especially in summer in this climate. The most meticulous care is required for every step in either procedure. A fluffy white ash secured at a low temperature, the absence of contaminations from chemicals, apparatus, or manipulations, and the correct proportion of the amounts of reagents used, particularly acids and oxidants, are primary essentials for either method.

All analyses were made in triplicate with a known quantity of iron added to and ashed with one of the three samples. In the case of urine the quantity of iron was added to each of the three samples; to one before ashing, to the other two before making to volume immediately preceding the development of the color. All results were discarded in which the recovery of added iron was not within certain limits, 97 to 103 per cent for food and feces, 99 to 101 per cent for urine, with the burden of experimental error falling wholly on the added iron. Table I illustrates these standards of accuracy. In one-half of all determinations the recovery was within the $\pm$ 1 per cent, in three-fourths it was within the $\pm$ 2 per cent limits of error.

TABLE I

Recovery of Added Iron

Case and period No.	Sample	Size	Added iron	Dilution and aliquot	Colorimeter readings	Total iron*	Average iron per sample	Recovery of added iron
			mg.	*cc.*	*mm.*	*mg.*	*mg.*	*per cent*
B, 1	Food 1	3 gm.	0.1	50:5	21.8	0.1858		102
	2			50:10	24.0	0.0814	0.0834	
	3			50:10	23.0	0.0854		
	Feces 1	0.5 gm.	0.1	100:5	31.7	0.2324		101
	2			100:10	27.7	0.1342	0.1316	
	3			100:10	28.4	0.1290		
	Urine 1	100 cc.	0.1	50:10	19.1	0.1047		100
	2		0.1	50:10	19.1	0.1047	0.0047	
	3		0.1	50:10	19.1	0.1047		
B, 2	Food 1	3 gm.	0.1	50:5	20.8	0.1958		102
	2			50:10	21.0	0.0947	0.0940	
	3			50:10	21.2	0.0934		
	Feces 1	0.5 gm.	0.1	100:5	28.8	0.2616		99
	2			100:10	23.6	0.1630	0.1630	
	3			100:10	23.6	0.1630		
	Urine 1	100 cc.	0.1	50:10	19.2	0.1043		100
	2		0.1	50:10	19.3	0.1036	0.0042	
	3		0.1	50:10	19.1	0.1047		
B, 3	Food 1	3 gm.	0.1	50:5	22.0	0.1840		103
	2			50:10	23.9	0.0820	0.0812	
	3			50:10	24.3	0.0803		
	Feces 1	0.5 gm.	0.1	100:5	27.5	0.2764		98
	2			100:10	21.4	0.1814	0.1784	
	3			100:10	22.0	0.1756		
	Urine 1	100 cc.	0.1	50:10	19.2	0.1043		99
	2		0.1	50:10	18.9	0.1058	0.0052	
	3		0.1	50:10	19.1	0.1047		

* The standard, set at 20 mm., contained 0.1 mg. of iron plus that in the reagents, diluted to 50 cc., of which a 10 cc. aliquot was used. Calculations are corrected for iron in reagents which varied 0.0090 to 0.0134 mg. per sample, depending on the dilution and aliquot used. The error from reagents is of less consequence if the unknown reads near to the standard. The 0.1 mg. of added iron was ashed with the urine in only the first of each

DISCUSSION

The iron balances for the twenty-three periods are shown in Table II. The average daily weight for dry food and feces and the volume for urine are included in this series because these aid in the interpretation of some of the findings.

TABLE II

Iron Balances in Pregnant Women (Daily Averages)

Case	Period No.	Wk. of preg- nancy	Collections*			Iron intake	Iron output			Balance
			Food	Urine	Feces		Urine	Feces	Total	
			gm.	*cc.*	*gm.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
A	1	12	350	2000	36	9.69	0.27	8.54	8.81	+0.88
	2	20	566	2500	43	15.73	0.22	10.23	10.45	+5.28
	3	28	591	2217	51	15.60	0.48	10.85	11.33	+4.27
	4	33	422	2298	44	14.60	0.46	10.30	10.76	+3.84
	5	39	358	3228	37	9.70	0.18	7.31	7.49	+2.21
B	1	16	446	1297	31	12.40	0.06	8.16	8.22	+4.18
	2	22	478	1300	37	14.96	0.06	12.06	12.12	+2.84
	3	29	499	1150	24	13.52	0.06	8.56	8.62	+4.90
	4	35	451	1500	28	11.68	0.08	8.68	8.76	+2.92
	5	39	462	1470	28	12.84	0.06	10.86	10.92	+1.92
C	1	27	382	893	28	12.83	0.09	10.75	10.84	+1.99
	2	33	392	1080	29	11.92	0.08	9.85	9.93	+1.99
	3	37	479	1400	27	15.47	0.10	11.13	11.23	+4.24
D	1	30	442	1465	34	18.52	0.14	15.88	16.02	+2.50
	2	34	487	2000	34	15.24	0.28	13.95	14.23	+1.01
	3	38	556	2200	50	15.51	0.32	17.40	17.72	−2.21
E	1	11	448	1159	32	18.73	0.06	15.45	15.51	+3.22
	2	21	579	1750	32	19.45	0.12	12.36	12.48	+6.97
	3	32	547	2200	33	16.30	0.13	11.29	11.42	+4.88
F		15	514	1847	33	19.27	0.11	13.70	13.81	+5.46
G		29	388	1280	25	14.64	0.12	10.25	10.37	+4.27
H		36	444	1008	33	13.01	0.09	9.57	9.66	+3.34
I		39	396	2500	31	16.95	0.36	14.76	15.12	+1.83

* Dry weights for food and feces. The urine volume represents collection plus a small amount of HCl, usually enough to total a round figure.

The intakes, representing usual home diets, ranged from 9.69 to 19.45 mg. with an average of 14.72 and a median of 14.86 mg. of iron daily. Two-thirds of the intakes fell within the range of 12.1 to 16.9 mg. per day. However, these figures are believed to represent amounts above that for the average dietary since in

four of these cases weekly servings of liver had been advised by the physician in charge.

The iron in the urine was very small, in most cases less than 2 per cent of that in the diet. The amount is well within the limits of experimental error for an intake-outgo study, and does not affect the final balance appreciably. The average daily volume of urine excreted ranged from 893 (Case C, Period 1) to 3228 cc. (Case A, Period 5), while the iron in the urine varied from 0.06 mg. (Case B, Period 3) to 0.48 mg. (Case A, Period 3), showing that the iron content was little related to the volume of urine. Neither was it in proportion to the iron in the diet. Relatively high iron excretion in the urine did seem to be characteristic of certain individuals (Cases A, D, and I), while for other subjects it ran comparatively low (Cases B and E).

These findings do not support the conclusion of Lintzel (18) that no iron whatever is excreted by the kidney. If the amounts reported for kidney excretion in this paper were due to faulty analytical methods, one would expect the quantity to be in proportion to the volume of urine, since 100 cc. samples were used regularly for the analyses, and would not expect the rather constant daily excretion shown by certain individuals from period to period in spite of comparatively wide variations in volumes of urine (Cases C and A).

The balances were surprisingly large in some cases and, with one exception, all were positive. The storage ranged from +6.97 to −2.21 mg. per day, amounting frequently to one-third of the iron ingested in the food (Case A, Period 2, Case B, Period 1, Case B, Period 3, and Case E, Period 2.)

Chart I shows the storage of iron in a graphic way. Horizontal lines indicate probable fetal demand as estimated by the Committee (3) and are calculated from the data of Hugounenq and of Camerer and Söldner. From Chart I it is evident that high storage rates occurred early in pregnancy, with a tendency to lower ones during the very last month. Whether this lower retention at the last is due to lessened demands of pregnancy as a whole including both maternal and fetal needs, or to lower iron ingestion, or is coincident with the decreased nitrogen retention which resulted from restricted protein intakes, cannot be determined from this series. The figures for fetal analyses showed great-

est increments during the last third of the period of gestation, particularly the last month. This requirement could possibly be met by marked increase in the reserves of iron in the liver or other tissues of the mother earlier in pregnancy, although Kojima's results on rats (10) do not support such a theory. In general, retentions during the first 6 months of pregnancy were in
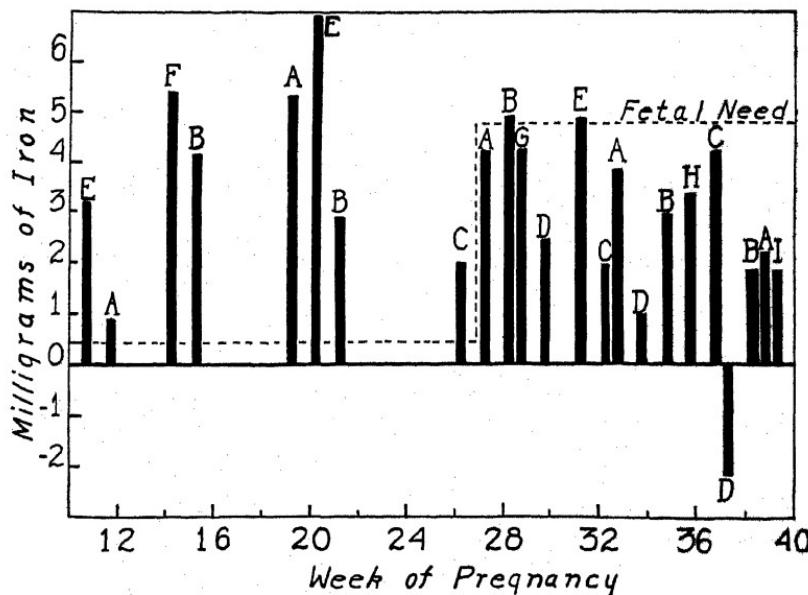
CHART I. Daily iron retention by nine women at different weeks of pregnancy. The dotted line represents probable fetal need as computed from published fetal analyses by the Sub-Committee on Nutrition for the White House Conference on Child Health and Protection.

excess of the calculated probable fetal demands, whereas during the last of the period they did not equal it, some falling far short.

Without doubt the early high iron retentions were for maternal benefits aside from immediate fetal needs. Thus, in Case A the storage rate was high at the 20th week following the period of nausea and semistarvation which had not completely ended by the 12th week. Case F was exhibiting a large appetite and probably carrying on extensive body repairs at the 15th week, although further studies were not available to tell what happened subsequently in this

subject. Case E is the most typical of a subject replenishing body reserves. She was known to have been anemic previous to the beginning of pregnancy, and to have added a total of 40 pounds to her body weight during the term, most of it in the early part. Furthermore, her balances for other elements, particularly phosphorus and nitrogen (11), showed high retentions. That neither the high intake nor the high storage continued to the end was shown by a later study, although our data do not show how soon the retentions ceased to be so abnormal. Nor have we record of how often conditions like that described for Case D below offset periods of high retention like this.

The high iron storage during Period 2 in Case E answers a question raised by Sandiford, Wheeler, and Boothby (19), as to whether or not the high nitrogen retention may not have been due to incomplete urine collections. (See Table I for average daily collections.) The iron in the urine was less than 1 per cent of the intake in this period, hence the iron balance depended very little on the urine collections. Yet this balance is the highest recorded for this series, just as the phosphorus and nitrogen balances were the highest of any reported. This supports our former conclusion that the excessive intake and retentions of this subject were in response to a physiological demand, apart from fetal needs, and that apparently the demand was satisfied in the early part of the pregnancy.

Case D, with the only negative balance, illustrates another important factor in iron metabolism. Sherman (2) called attention to the drastic effects of a slight upset in digestion on an iron balance. At the time of this negative balance, the daily average of dry feces rose from 34 gm. in previous periods to 50 gm. in Period 3 (Table I). Original records show regularly two stools a day for this period in contrast to one daily during previous observations.

Differences in diet tend to explain most of the other individual variations in the retention of iron. Table III summarizes in a crudely quantitative way some of the dietary factors likely to affect iron metabolism. The average daily amounts of meats excluding fish, of liver, eggs, of green leafy vegetables, including string beans, and of milk consumed by each subject during each period have been tabulated. This takes no account of fruits high

in iron, or of those such as apricots and peaches known to favor hemoglobin formation.

The abundant egg and green vegetable content of the diets of Case A is associated with relatively high storage in spite of the absence of meats. Case B's best retention came during Period 3 when the diet was most adequate in eggs, green vegetables, and

TABLE III

Chief Protein- and Iron-Containing Foods in Diets Chosen by Women

Daily Averages

Case	Period No.	Meats	Liver	Eggs	Green vegetables	Milk
		gm.	*gm.*	*gm.*	*gm.*	*gm.*
A	1			23	14	200
	2			80	138	863
	3			16	77	734
	4	3		39	64	602
	5			6	95	575
B	1	36				538
	2	41		10	2	366
	3	75		18	20	192
	4	44				363
	5	41		18		73
C	1	77		45	15	132
	2	79	21	60		434
	3	73	17	69		503
D	1	13	33		135	538
	2				65	475
	3			9	90	269
E	1	63		50	7	889
	2	20	9	8	77	667
	3			30	62	733
F		58		79	111	211
G		72		26	69	118
H		5	22	18	44	227
I		54	25	67	156	356

meats. Case C stored most in Period 3 when more fruits were included in a diet adequate in meat and eggs but notably lacking in green vegetables. On the other hand, the very high green vegetable and consequently high iron content of the diet of Case D did not make up for the almost complete absence of meats and eggs.

Cases G and H ranked comparatively higher in iron retention, as a result of meat and liver with a reasonable amount of green vegetables in the diet, than they did with respect to calcium storage which was poor on account of the low milk intake.

The level of storage required for probable fetal needs, as indicated in Chart I, is based in the early part of gestation on calculations from Hugounenq's figures which are doubtless much too low as shown by Camerer's data on iron content of the new-born. The additional maternal needs for iron for the entire period may be estimated by applying the average 0.006 percentage for iron in the adult body to the maternal gain in weight in excess of that due to the fetus, assuming the lack of iron in amniotic fluid is partly compensated by the large proportion of blood in the placenta. The average gain for this group of women (11) was 21 pounds in excess of fetal weight. This would call for over 0.5 gm. of iron in addition to nearly 0.4 gm. for the fetus, or a total of 0.9 gm. to be supplied from food or body reserves. An average daily storage of 3.2 mg. for the entire period of gestion would cover this need. Case A averaged 3.3 mg., Case B, 3.4 mg. during the five scattered periods observed for each of these, whereas the average storage for the entire group was 3.2 mg. of iron daily.

It is probable, therefore, that only the best diets were supplying the iron demands of pregnancy but that the poorest, particularly those of Case D, were inadequate.

SUMMARY

Twenty-three iron balances on women at different stages of pregnancy are reported.

With intakes varying from 9.69 to 19.45 mg. of iron per day, the retentions varied from +0.88 to +6.97 with one exception, a negative balance of −2.21.

Under fairly ideal conditions of diet and well being, it seems possible for the maternal organism to assimilate during the period of pregnancy enough iron from food to supply the new born infant with the needed reserves.

The quality as well as the quantity of iron intake, the physiological demands of pregnancy, and slight upsets in digestion seemed to be important factors in iron retention.

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DESIGN AND USE OF A GLASS CAGE IN ANEMIA STUDIES*

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The desire to eliminate all possible chance for contamination by inorganic elements in studies of nutritional anemia has brought the use of wire cages into some disfavor. Consequently various investigators (1–6) have made use of glass cages. We, ourselves, have felt the necessity, in a detailed study of the rôle of manganese in nutrition (7), for the use of other than metallic cages and accordingly have constructed some of glass. They are inexpensive, actually costing less than our wire cages; they are fairly light, yet rigid; they can be readily lifted and moved about by one person; and, lastly, they are durable. In using thirteen of them for more than a year we have not been obliged to make any replacements of parts. Essentially the cage (Fig. 1) consists of four walls of glass window-panes with a floor of suitably spaced glass tubing supported by a metal frame standing on four legs. The glass walls are capped with a hinged metallic cover, and, for utility's sake, the entire unit is placed in a pan for the collection of excreta.

The supporting frame and top, as well as the pan in which the cage rests, are constructed of galvanized sheet iron. The frame is $18\frac{1}{4}$ inches long, $14\frac{1}{2}$ inches wide, and 6 inches high, inside dimensions. The ends, instead of being made of material 6 inches wide as the sides are, are constructed of 9 inch material, the extra 3 inches being turned up to make a deep groove for holding the end panes of glass in upright position. Holes $\frac{1}{4}$ inch in diameter and spaced $\frac{1}{2}$ inch apart, from center to center, are drilled in each side of the frame at a distance of 2 inches below the upper edge. These

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holes accommodate 6 mm. glass tubing, 15½ inches long, which, extending from one side of the cage to the other, forms the false open bottom. Each tube is held in place by two rubber bands slipped over the extending ends. The glass panes forming the sides rest on this false bottom. Their lower ends are held in place by the metal frame on the outside, and by the end pane within. Their upper ends fit into a groove in the metal top. This top, in addition to imparting rigidity to the cage, also supports
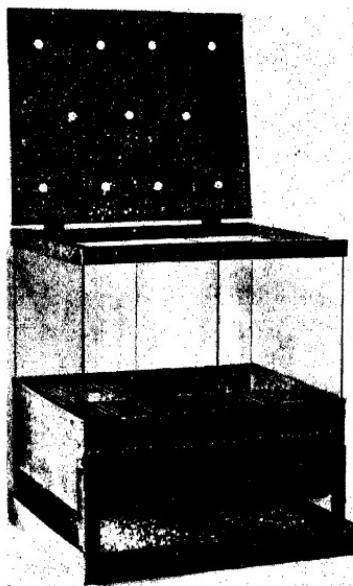
FIG. 1. A glass cage for nutritional studies

the hinged cover. Two 1 inch metal strips, the ends of which are soldered to diagonal corners at the lower edge of the metal frame, give to the supporting structure additional strength.

The entire cage can be divided into two compartments by a pane of glass, which is supported below the floor by two U-shaped metal strips soldered on the sides of the frame, and is held in position at the upper edge by a special groove in either side of the metal cover. Each half of the cage can again be divided into two by insertion of a pane of glass, thus giving four small compartments

having a floor area of 9 by 7 inches each. These panes are held in position at the top by small notches cut into the top frame and at the bottom by M-shaped bends in a few of the glass tubes which form the floor. The legs raise the cage 4 inches above the floor proper and thus provide space for free circulation of air. The pan in which the cage rests is 19 inches long by 15 inches wide, and 2 inches deep. This is kept partially filled with wood shavings treated with an antiseptic, for absorption of urine.

The glass housing of the cage proper is built of double strength glass window-panes; the side panes are 18 by 12 inches; the ends, 16 by 14 inches; and the partition when divided into two, 15¼ by 14 inches. These sizes give a distance of 12 inches from the floor of the cage to the top, which, except for an occasional, very active rat, is sufficient to keep the animals from jumping to the top and making any contact with metal whatsoever. If it should be considered desirable to prevent all possibility of this the frame is quite strong enough to allow increasing the height.

It is unnecessary to dissemble the cage for cleaning, since its weight, size, and rigidity permit its being easily carried to a sink where, with the aid of a brush and a stream of hot water, it may be cleaned readily.

Glass versus Wire Cages for Use in Production of Anemia—After starting our studies on manganese it was decided to compare the relative merits of a glass cage with our ordinary wire cages for use in a study of nutritional anemia. Accordingly eight young rats, in which copper and manganese storage had been prevented during the suckling period as described in another paper (7), were divided into two groups so as to equalize the sexes, weights, and hemoglobin values. One group was placed in the glass cage and the other in a quarter section of one of our regular wire cages, which provided about the same floor space as the glass cage. A wire screen was used as a false bottom for the wire cage. Both groups were supplied with milk *ad libitum* plus 0.1 mg. of iron, as the purified chloride, per animal daily.

Inspection of Chart I, which presents the results of this experiment, reveals that all the animals in the wire cage showed a diminution of hemoglobin which in most cases began immediately, and three of the four died before the end of 10 weeks. To our surprise, animals in the glass cage fared much better. All showed

progressive hemoglobin regeneration and slow but continuous growth throughout the greater portion of the period.

Effect of Change from Glass to Wire Cages and Vice Versa— Further evidence that anemia is more easily produced in wire than in glass cages was obtained upon the transfer of twenty-four rats from cages of the latter type to wire cages. For 10 weeks these animals had been in individual glass cages and had received milk *ad libitum* plus 0.1 mg. of iron and 0.001 mg. of copper per animal daily. In addition, eighteen of them had received manganese at either a 0.0041 or 0.25 mg. level. In the wire cages the
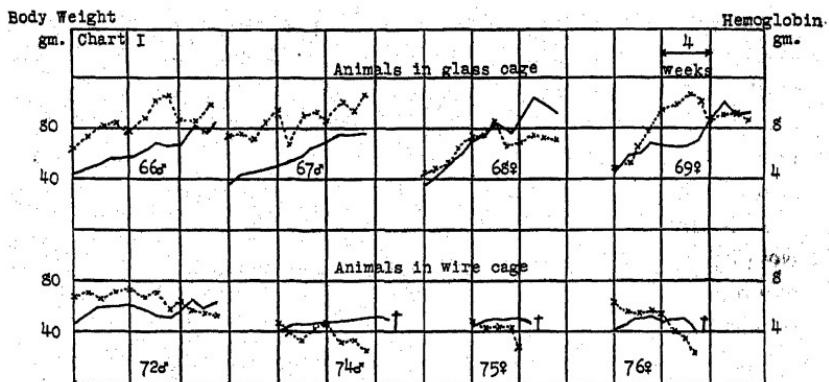
CHART I. Growth and hemoglobin curves of rats receiving milk *ad libitum* plus 0.1 mg. of iron daily as affected by type of cage. It is evident that the animals in the glass cage fared much better than those in the wire cage. The solid line represents body weight, while the broken line represents hemoglobin in gm. per 100 cc. of blood. The dagger indicates death.

animals were grouped according to their respective supplements and were continued on them.

The growth and hemoglobin curves for eight of these animals, which are representative of the entire lot, are given in Chart II. The performances during the month preceding the transfer from glass to wire cages have been included in order that the effect of transfer may be evident. (The data for this entire period have been detailed elsewhere (7).) During the 8 weeks that they were in the wire cages all twenty-four rats showed slight to pronounced decreases in hemoglobin, and growth was often noticeably retarded. Moreover, the animals became anemic in appear-

ance, and they seemed to be in worse condition than their weights indicated. At the end of this period they were again placed in
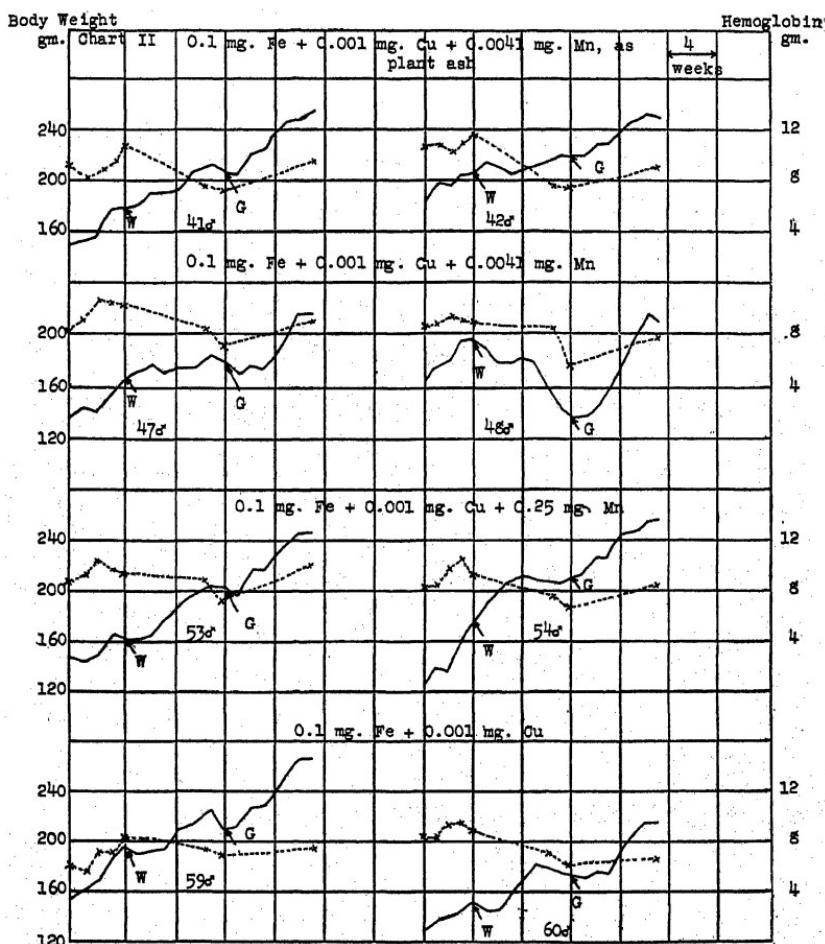
CHART II. The effect of changing rats from glass to wire cages and *vice versa*. The animals were transferred to wire cages at the point marked *W* and back to glass ones at the point marked *G*. The solid line represents body weight, while the broken line represents hemoglobin in gm. per 100 cc. of blood.

glass cages; this time the six animals of each group were placed in one of the 18 by 14 inch cages. Two of the rats continued to lose weight and died after being in the glass cages for 1 and 5

weeks, respectively. Of those remaining, however, sixteen showed definite hemoglobin regeneration during the next 7 weeks, none of them became more anemic, and in most instances the rate of growth was increased.

Data similar to the above were obtained on another group of seven rats which were reared by mothers on an exclusive milk diet. At weaning age they were placed in a wire cage and provided with milk *ad libitum* plus 0.1 mg. of iron and 0.001 mg. of copper per animal daily. For the sake of brevity, growth and hemoglobin curves for these animals have been omitted. Suffice it to say that all became quite anemic during 7 weeks of such treatment, but upon being transferred to a glass cage four of them showed marked improvement in growth, hemoglobin content, and general appearance. The other three presumably were too weak to respond to the change; two of them died soon and the other seemed barely to maintain life during the next 7 weeks.

Effect of Washing Glass Cages—It is apparent from the preceding data that there were marked differences in the results obtained with animals confined in the two types of cages. Although there were several factors which, presumably, might cause these differences, the one which seemed most likely to be responsible was that of increased coprophagy subsequent to greater adhesion of excreta to the tubes which formed the false bottoms of the cages. Other possibilities included differences in temperature and air currents as well as the presence of some toxic factor in the wire screen, which the animal might obtain in minute amounts.

In order to study these possibilities two separate series of experiments were carried out. For the first, two groups of four rats each were selected from young in which storage of copper had been prevented during suckling, as explained in another paper (7), and were placed in two glass cages. A wire screen was placed in one cage for a false bottom, raised over the plane of the glass tubes, thereby eliminating differences in temperature and aeration in the two cages—possible factors in the previous experiments. Neither cage was washed during the experimental period. Later, two additional groups of six animals each were selected as the ones above and placed in two glass cages, one of which was provided with a wire screen for a false bottom. The other cage, which was left unchanged, was washed daily with water redistilled

from Pyrex glass.   In these experiments the iron supplement was fed at a level of 1.0 mg. per 100 cc. of milk.

For the sake of brevity the results of these two series of experiments have been combined in one chart; *viz.*, Chart III.   It will
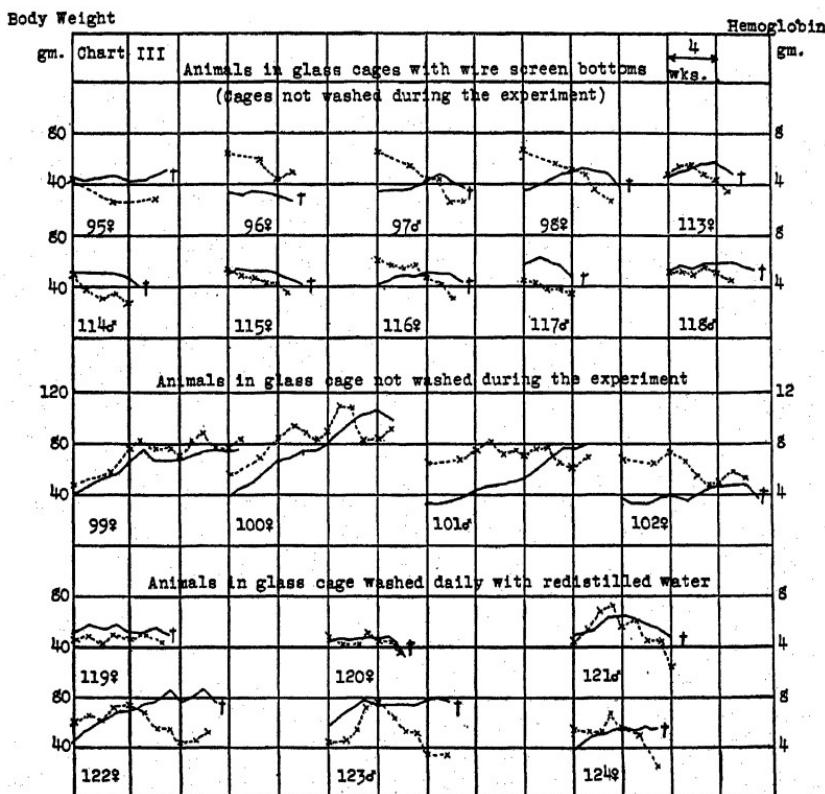
CHART III. When adherent excreta were removed from the glass cages by washing daily with redistilled water, animals succumbed to anemia almost as readily as when kept in wire cages. The solid line represents body weight, while the broken line represents hemoglobin in gm. per 100 cc. of blood. The dagger indicates death.

be noted that all of the rats in the cages provided with wire screen bottoms soon became anemic and died within 4 to 8 weeks.   Of the four animals in the regular glass cage which was not washed, only one died and at the end of 13 weeks the others showed no signs of succumbing to anemia.   However, all of the rats in the glass cage

which was washed daily died within 6 to 11 weeks; the average was 8.2 weeks. Thus even with such precautions these animals were able to survive longer than their litter mates which were kept in similar cages provided with wire bottoms.

The results presented above show conclusively that the differences in behavior of rats kept in wire and glass cages, respectively, are attributable mainly to the greater surface offered by the bottoms of the latter for retention of minerals in the excreta. They amply illustrate how carefully conditions must be controlled during investigations of the factors which affect the welfare of the animal. Such precautions are doubly necessary when studying the rôle of substances physiologically active in such minute amounts as copper.

SUMMARY

The construction of a glass cage suitable for rats has been described in detail.

Glass cages proved less suitable for our anemia studies than cages constructed of galvanized iron wire.

The difference in response in the two types of cages has been shown to be due largely to increased coprophagy subsequent to greater adhesion of excreta to the tubes which formed the bottoms of the glass cages.

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THE ACTION OF SULFITE UPON CYSTINE*

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From discussions (4) of the Folin-Looney procedure (3) for
estimating cystine it might be inferred that the action of sodium
sulfite upon cystine consists of a reduction in the commonly ac-
cepted sense.

$$R—SS—R + Na_2SO_3 + H_2O = 2R—SH + Na_2SO_4$$
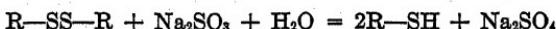
The validity of this inference has, however, recently been thrown
open to doubt by the statement (8) that the color intensity given
in this test by cystine is only one-half of that given by cysteine.
A similar doubt exists in the case of Sullivan's test (11) for cystine,
in view of his observation that "the reduction may indicate only
50 per cent of the theoretical, but usually it runs about 75 per
cent." This may be explained by the fact (7, 10) that cystine
undergoes a double decomposition with potassium cyanide.

$$R—SS—R + KCN = R—SK + R—SCN$$

Examination of the reaction between cystine and sodium sulfite
has shown it to take an analogous course.

$$R—SS—R + Na_2SO_3 = R—SNa + R—SSO_3Na$$
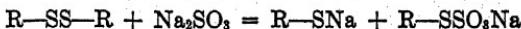
Sulfate is not produced to any appreciable extent. After faint
acidification of the reaction mixture and removal of the excess of
sulfur dioxide by boiling, the reducing power towards iodine has
been found to correspond to 95 to 128 per cent of the amount of
cysteine calculated from the above equation. As will be seen, the
production of more than the anticipated values is ascribable to
hydrolysis of the sodium S-cysteinesulfonate.

The formation of cysteine has been confirmed by shaking an alkaline solution of cystine and sulfite with benzyl chloride for 5 hours. Benzyl cysteine was isolated in yields of 60 to 80 per cent of the calculated amount. Control experiments carried out without sulfite yielded no detectable benzyl cysteine under these conditions; a yield of less than 5 per cent could be isolated after 5 days treatment.

The isolation of the thiosulfuric acid derivative involves the preliminary removal of cysteine; this was accomplished (9, 14) by acidifying the solution of cystine and sulfite by means of acetic acid and adding two molecular proportions of copper sulfate previously reduced to the cuprous condition by sodium sulfite and acetic acid. An insoluble complex salt, of a composition best represented by the formula $6C_3H_6O_2NSCu \cdot 2Cu_2SO_3 \cdot Cu_2SO_4$, precipitated; this product contained about one-third of the nitrogen originally taken as cystine. The filtrate was intensely blue, and no apparent change occurred when a test portion was treated with an excess of sodium carbonate or hydroxide. After the addition of barium hydroxide and barium acetate until no further precipitation occurred, the solution was substantially free from sulfate and sulfite ions; practically all of the copper and about 2 per cent of the original amount of nitrogen were carried down in the precipitate. The colorless, faintly acid filtrate was evaporated to dryness under reduced pressure and extracted with cold 95 per cent ethyl alcohol, to remove the sodium acetate. The residue after one recrystallization yielded sodium S-cysteinesulfonate, $2COOH \cdot CH(NH_2) \cdot CH_2SSO_3Na \cdot 3H_2O$, in almost pure condition, only traces of copper being present as impurity.

The corresponding ammonium salt was obtained, in the form of needles containing no water of crystallization, by treating an ammoniacal solution of cystine with a large excess of ammonium sulfite and allowing the latter to volatilize at room temperature in the presence of air. This salt appeared to be the only product. It therefore seems probable that the cysteine initially formed underwent autoxidation to cystine, and that this in turn reacted with further quantities of ammonium sulfite.

These salts are stable in water and in cold dilute hydrochloric acid. In alkaline solution they decompose with formation of sulfite and of substances which reduce the Folin-Looney phos-

photungstic acid reagent. For this reason, relatively concentrated aqueous solutions slowly develop a blue color when treated successively with sodium sulfite, sodium carbonate, and phosphotungstic acid. On boiling with N (or more concentrated) hydrochloric acid, hydrolysis slowly takes place, with formation of sulfuric acid and cysteine.

$$COOH \cdot CH(NH_2) \cdot CH_2SSO_3H + H_2O = COOH \cdot CH(NH_2) \cdot CH_2SH + H_2SO_4$$
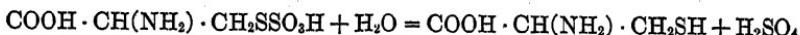
This behavior towards acids resembles that observed with sodium ethylthiosulfate (1), and, as in that case, probably takes a more complicated course (6). Somewhat less than the calculated amount of sulfuric acid was recovered, and not only was some of the cysteine oxidized to cystine during the hydrolysis, but appreciable loss of optical activity occurred.

On oxidation with bromine water, sulfuric acid and cysteic acid are formed.

$$COOH \cdot CH(NH_2) \cdot CH_2SSO_3H + 3Br_2 + 4H_2O =$$
$$COOH \cdot CH(NH_2) \cdot CH_2SO_3H + H_2SO_4 + 6HBr$$

In this case, the yield of sulfuric acid was substantially quantitative, and the optical rotation of the final reaction mixture indicated that cysteic acid was the only other product.

While sodium hydroxide causes the liberation of sulfite from the above salts, in the same manner as from sodium ethylthiosulfate (5), the prolonged action of barium hydroxide in excess upon the ammonium salt led, in one experiment, to the formation of barium thiosulfate, presumably by a more extensive disruption of the molecule. The loss of optical activity of cystine in alkaline solution (Table I) is temporarily inhibited by the addition of sulfite. A solution containing 0.025 mol of cystine, 0.10 mol of sodium sulfite, and 0.08 mol of sodium hydroxide in 145 cc. had an apparently constant specific rotation. Addition of ten molecular proportions of sodium sulfite to a 0.025 M solution of cystine in N sodium hydroxide caused an apparent stabilization of rotation, which extended over 2 days (Table II). This effect is probably due, at least partly, to the greater stability towards alkali of cysteine than cystine (2).

The rotation of solutions of cystine in alkaline sulfite varies to some extent with the concentration of alkali and with the propor-

TABLE I

Change in Rotation of 0.025 M Cystine in N NaOH at 25°

Time	$[\alpha]_{546}$	Time	$[\alpha]_{546}$	Time	$[\alpha]_{546}$
hrs.	*degrees*	*hrs.*	*degrees*	*hrs.*	*degrees*
0.25	−103	115	−67	361	−22
3.5	−98	145	−54	385	−15
19	−95	192	−51	430	−11
43	−89	218	−41	482	−6
70	−82	312	−31	529	−4

TABLE II

Change in Rotation of 0.025 M Cystine in 0.25 M Na_2SO_3 in N NaOH at 20–25°

Time	$[\alpha]_{546}$	Time	$[\alpha]_{546}$	Time	$[\alpha]_{546}$
hrs.	*degrees*	*hrs.*	*degrees*	*hrs.*	*degrees*
0.17	−81	99	−77	334	−34
0.67	−80	152	−71	360	−30
1.67	−80	199	−61	388	−24
6.0	−80	217	−57	430	−18
21.5	−79	266	−46	484	−12
30.8	−80	286	−44	621	−4
49.6	−79	312	−39		

TABLE III

Rotations of 0.025 M Cystine with Equivalent and Twice Equivalent Amounts of Alkali, in Presence of Various Proportions of Sodium Sulfite and Sulfate

Na_2SO_3	Na_2SO_4	$[\alpha]_D$	
		NaOH, 0.05 M	NaOH, 0.10 M
M	*M*	*degrees*	*degrees*
0	0	−92	−83
0	0.50	−82	−78
0.05	0.45	−79	−73
0.10	0.40	−77	
0.15	0.35	−73	−70
0.20	0.30	−71	−67
0.25	0.25	−70	−66
0.35	0.15	−66	−65
0.50	0	−64	−63

tion of sulfite (Table III). This fact, taken in conjunction with the effect, discussed above, of alkali upon cysteinesulfonate, may be taken as indicative of the reversible nature of the reaction between cystine and sulfite. That the equilibrium can be grossly disturbed is shown by changes in rotation on treatment with alkaline stannite, which appears to cause almost complete reduction to cysteine. Acidification of the cystine-sulfite solution in the cold causes only a slight increase in levorotation; this falls markedly after boiling, apparently owing to hydrolysis.
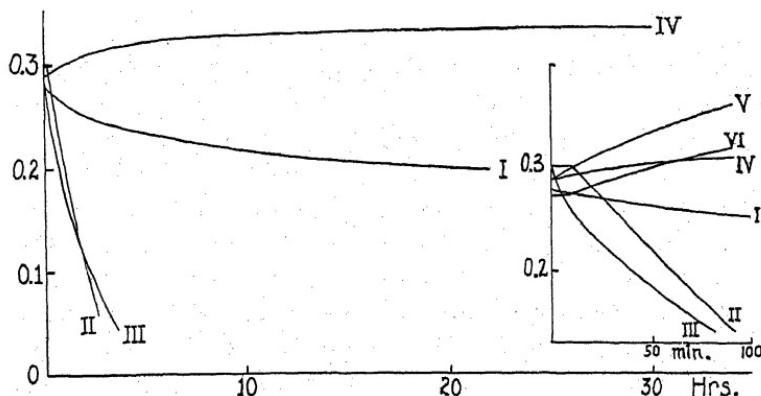
FIG. 1. Intensities of absorption of light of wave-length 668 $m\mu$ by 5 mm. layers of solutions prepared from 2 mg. quantities of cystine in a total of 100 cc. Curve I shows the results obtained with the Folin-Marenzi method (diluent Na_2SO_3) with the cover-glass; Curve II, Folin-Marenzi method (diluent Na_2SO_3) without the cover-glass; Curve III, Folin-Marenzi method (diluent H_2O) without the cover-glass; Curve IV, Tompsett method (diluent Na_2SO_3) with the cover-glass; Curve V, Tompsett method (diluent Na_2SO_3) without the cover-glass; Curve VI, Tompsett method (diluent H_2O) without the cover-glass.

The cysteinesulfonates differ from cystine in having higher levorotations in pure water than in either acid or alkali. The ammonium salt has a somewhat higher molecular rotation in water ($-23,700$) than the sodium salt ($-21,700$); the difference in N acid ($-12,960$, $-12,425$) is less than in N alkali (-6650, -5975).

The observation of Sullivan that in his test (11) cystine gives 50 to 75 per cent as intense a color as cysteine has been checked by finding a value of 168 per cent for the latter under standard conditions. Moreover, in general agreement with the observation of

Mirsky and Anson (8), color intensity values of about 175 per cent of that of cystine were obtained in the Folin-Marenzi process (4), both with preformed cysteine hydrochloride and with acid solutions of cystine reduced by contact with zinc filings in the cold.[1] Owing to the rapidity with which the color fades on exposure to air, the accuracy of the method of Folin and Marenzi depends largely upon the degree of correspondence in the treatment of the unknown and standard colored solutions, subsequent to the development of the color. In Fig. 1 are shown the changes with time of the optical densities for light of wave-length 668 $m\mu$, determined spectrophotometrically, of standard solutions in an open Schultz cell and in the same cell covered with a glass plate. The effect of dilution with water rather than with 3 per cent sodium sulfite (4) is also shown. For comparison, curves are included of the changes in intensity of the color developed from the same amount of cystine by the procedure recently described by Tompsett (12). It will be seen that the initial color intensities of both procedures nearly coincide, but that the Tompsett process leads to a color which intensifies to a steady value. The enhanced rates of intensification in the Tompsett colors exposed to air can be explained by evaporation from the open Schultz cell.

<center>EXPERIMENTAL</center>

<center>*Action of Sodium Sulfite on Cystine in Alkali*</center>

To a solution of 6.00 gm. (25 mM) of cystine in 40 cc. of 2 N sodium hydroxide were added 100 cc. of a solution containing 12.6 gm. (100 mM) of sodium sulfite. The resulting mixture, the volume of which was 145 cc., showed $\alpha_{546}^{23} = -3.98°$ (2 dm.), whence $[\alpha]_{546}^{23} = -96.4°$. This value remained unchanged during 29 days at 20–25°. After 1 hour, a 10 cc. portion was treated with excess of barium chloride; the precipitate was collected and washed by centrifugation, and treated with hydrochloric acid. The undissolved residue of barium sulfate amounted to only 5 mg. more than was obtained in a similar experiment from a corresponding amount of sodium sulfite. A 5 cc. portion was acidified

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[1] Values of about 200 per cent have been observed by Vickery and White (15) with cysteine solutions prepared by reducing cystine with tin and sulfuric acid and removing the dissolved tin with hydrogen sulfide.

with hydrochloric acid, boiled until free from sulfur dioxide, and titrated with iodine, of which 0.818 milli-equivalent was required. Since the 5 cc. contained 0.8615 mol of cystine, this corresponds to a 47.5 per cent conversion of cystine into cysteine. In similar experiments in which a considerable excess of hydrochloric acid was employed, higher reducing values were obtained, the greatest corresponding to 64 per cent conversion.

A mixture of equal volumes of 0.05 M cystine in N sodium hydroxide and 2 M sodium sulfite in N alkali had $[\alpha]_{546} = -81.7°$. When this mixture was treated with an equal volume of M sodium stannite in N NaOH, the rotation became $[\alpha]_{546} = -10°$. Cystine with alkaline stannite under similar conditions, but in the absence of sulfite, had $[\alpha]_{546} = 0°$, changed to $-14.6°$ on acidifying with hydrochloric acid. Striking changes in rotation were also observed on treating cystine-sulfite mixtures with acid. A solution of 2.40 gm. (10 mM) of cystine, 4 gm. of sodium carbonate, 2 gm. of sodium sulfite, and 0.8 gm. of sodium hydroxide in 100 cc. had $[\alpha]_{546} = -91.7°$. On acidifying with hydrochloric acid the rotation became $[\alpha]_{546} = -97.8°$. On diluting and expelling sulfur dioxide by boiling, the rotation fell to $-61.4°$. The resulting solution was titrated with iodine, when 12.75 milli-equivalents were required. The oxidized solution then had $[\alpha]_{546} = -217°$, a value approximating that of cystine in hydrochloric acid.

Benzyl Cysteine

A solution of 6.00 gm. (25 mM) of cystine and 6.4 gm. (51 mM) of sodium sulfite in 120 cc. of N sodium hydroxide was shaken with 6.5 cc. of benzyl chloride for 5 hours at room temperature. After extraction with ether, the alkaline solution was acidified with 5 cc. of acetic acid. The crystalline product (leaflets) was collected, washed with water, and dried. It weighed 3.55 gm., contained 8.5 per cent (44.2 per cent of the original) nitrogen, and melted at 215° (uncorrected). Traces of unchanged cystine were removed by recrystallizing from 2 N hydrochloric acid. The hydrochloride which separated was readily converted into free benzyl cysteine melting at 216–217° (uncorrected) by washing with water. The filtrate from the crude benzyl cysteine contained no appreciable quantity of sulfate.

Action of Benzyl Chloride on Cystine in Alkali

A solution of 6.00 gm. (50 milli-equivalents) of cystine in 120 cc. of N sodium hydroxide was treated with 6.5 cc. of benzyl chloride and allowed to stand at room temperature, with occasional shaking, for 5 days. The water-insoluble products were removed by shaking with ether and the alkaline solution was acidified with acetic acid. The insoluble product was filtered off, washed with water, and dissolved in normal hydrochloric acid to 200 cc. This solution contained 28.1 milli-equivalents of nitrogen (56 per cent of that taken) and 3.34 gm. (27.8 milli-equivalents) of cystine (determined by the method of Tompsett). 180 cc. of it were evaporated to 25 cc. and chilled. The crystalline hydrochloride was washed successively with concentrated hydrochloric acid, water, and alcohol. The resulting free benzyl cysteine (0.40 gm.) melted at 216° (uncorrected) and contained 6.34 per cent of nitrogen (calculated, 6.63 per cent).

The filtrate and washings were united and evaporated nearly to dryness; the residue was dissolved in 2 to 3 cc. of water and treated with 5 cc. of acetic acid. On chilling, the bulk of the dissolved hydrochloride separated in microscopic needles which were filtered off and washed with acetic acid. This product formed a clear solution in 3 to 4 cc. of water, but on diluting to 15 cc., crystallization rapidly occurred, with formation of hexagons and needles of partially racemized cystine.

When the above procedure was carried out with only 5 hours for the reaction in place of 5 days, no benzyl cysteine could be isolated; the bulk of the cystine was recovered in a slightly racemized condition.

Cuprous Cysteine-Sulfite-Sulfate

To a solution of 12.5 gm. of crystallized copper sulfate (0.05 mol) in 100 cc. of water were added 6 cc. of acetic acid (0.1 mol) and a solution of 6.4 gm. of sodium sulfite (0.051 mol) in 50 cc. of water. A yellow solid separated. The resulting suspension was added, in several portions, to a solution of 6.00 gm. of cystine (25 mM) and 6.4 gm. of sodium sulfite (51 mM) in 100 cc. of 0.5 N NaOH, to which, after 20 hours standing, 1 cc. of acetic acid (17 mM) had been added. The first portions dissolved to form a colorless solution, which on further additions continued clear but became almost

black in color; after half of the cuprous suspension had been added, further addition caused the separation of a pale green solid. This was filtered off, well washed with cold water, and dried *in vacuo*. In two such experiments 5.40 gm. containing 4.21 per cent (16.2 milli-equivalents) of nitrogen and 5.00 gm. containing 4.17 per cent (14.9 milli-equivalents) of nitrogen were obtained respectively. The dry product was a dark green, amorphous, hygroscopic solid which attained constant weight only after about 50 hours at 115–120°, becoming considerably darker during desiccation. The moisture content was 8.5 per cent.

Analysis (*on Dry Basis*)—C 12.26, H 1.79, Cu 44.5 (by precipitation), 45.0 (by ignition), N 4.67, total S 16.4, sulfite S 3.63, sulfate S 1.87

Calculated for $6C_3H_6O_2NSCu \cdot 2Cu_2SO_3 \cdot Cu_2SO_4$. C 12.41, H 2.07, Cu 43.85, N 4.83, total S 16.58, sulfite S 3.68, sulfate S 1.84

The substance is readily soluble in dilute hydrochloric acid, yielding a very pale yellow (almost colorless) solution, which tends to become turbid on standing in air. After removing the copper from such a solution by means of hydrogen sulfide, a specific rotation of $-158°$ was observed, rising to $-180°$ after titration with iodine. These values are calculated on the basis of the amounts of cysteine and cystine corresponding to the observed nitrogen content. The iodine titration indicated that 20 per cent of the cysteine had become oxidized to the disulfide form, possibly during the drying.

With dilute sulfuric acid the compound yields a light yellow, almost insoluble substance. This was not examined further but is probably identical with the precipitate, cuprous cysteine, described by Vickery and White (14).

Sodium S-Cysteinesulfonate

The blue filtrate from the cuprous cysteine was treated with 32 gm. of crystallized barium hydroxide (0.10 mol). The greenish precipitate (27.2 gm.) which contained the bulk of the copper, also contained 1.013 milli-equivalents of nitrogen. The almost colorless filtrate was weakly acidified with acetic acid and treated with barium acetate solution until no further precipitation took place. The colorless filtrate was concentrated under reduced pressure to a volume of 100 cc., filtered, and freed from a small

amount of dissolved barium by the addition of exactly the necessary quantity of sulfuric acid. It was then evaporated to dryness under reduced pressure. The residue was warmed with about 200 cc. of 95 per cent ethyl alcohol; the alcoholic solution was decanted and the insoluble portion washed twice more with 100 cc. portions of alcohol. The alcoholic solution deposited a small quantity of a substance which crystallized in short, crossed needles; this was not further examined. The alcohol-insoluble residue was taken up in a small amount of water, and the filtered solution was treated with 2 to 3 volumes of cold alcohol. Two liquid layers resulted; from the upper, a further small amount of the crossed needles deposited on the walls of the vessel, together with some transparent crystals of almost circular appearance, seemingly cubes with truncated corners. Indications were secured that these crystals consisted of the di-sodium salt of the cysteinesulfonic acid. They were, however, not closely investigated.

The greater part of the product separated from the lower layer in the form of colorless rectangular blocks, very readily soluble in water, insoluble in alcohol. These crystals were analyzed after drying in air at room temperature.

Analysis—Loss at 105–110° *in vacuo* 11.13, N 5.63, S 24.8, Na 9.85

Calculated for $C_3H_6O_5NS_2Na \cdot 1\frac{1}{2} H_2O$. H_2O 10.80, N 5.60, S 25.6, Na 9.20

Rotation—$[\alpha]_D^{23} = -86.8°$ (4.73 per cent in water), $-49.7°$ (2.36 per cent in N HCl), $-23.9°$ (2.36 per cent in N NaOH)

A sample on oxidation with bromine water yielded sulfate sulfur 11.85 per cent (calculated value 12.80 per cent). In another experiment, 0.3514 gm. was dissolved in 5 cc. of water and titrated with bromine in dilute HCl. 7.82 milli-equivalents were required (calculated, 8.22 milli-equivalents). The resulting solution was evaporated to complete dryness and dissolved to 10 cc.; this solution had a rotation of $\alpha_D^{23} = +0.45°$ (2 dm.). Accepting the recorded specific rotation $[\alpha]_D = +8.66°$ for cysteic acid, the calculated value would be $+0.41°$. The solution was diluted to about 100 cc. and boiled with excess of freshly precipitated copper carbonate; the hot solution was filtered and concentrated to 4 cc. when blue crystals appeared. After cooling, these were collected, washed with cold water, and dried at 100°, when they gave analytical values agreeing satisfactorily with those for the copper salt of

cysteic acid: N 5.75, Cu 25.52. Calculated for $C_3H_7O_6NSCu$. N 5.63, Cu 25.57.

The action of hydrochloric acid is illustrated by the following experiment. A solution of 0.1225 gm. of the salt in 4 cc. of 5 per cent barium chloride was treated with 1 cc. of approximately 10 N HCl, and the clear solution heated in a loosely stoppered vessel at 100°. Separation of barium sulfate became appreciable after 15 minutes. Heating was continued for 22 hours; the precipitate weighed 0.1045 gm. (calculated 0.1103 gm.). The filtrate was concentrated to 6.8 cc., and then had $\alpha_D^{23} = -1.65°$ (2 dm.), whence $[\alpha]_D = -95.3°$ on the basis of 58.8 mg. of cystine. Assuming the specific rotation $[\alpha]_D = +16°$ for cysteine in HCl (13), the rotation, calculated for a 100 per cent yield of cysteine, should have been $\alpha_D = +0.28°$. The optical data (based on the observed specific rotation $[\alpha]_D = -209°$ for the cystine employed in the experiments) thus indicate a mixture containing almost exactly equal amounts of cysteine and cystine. Titration with iodine showed a reducing power of 0.254 milli-equivalents (51.8 per cent of the calculated value). The oxidized solution, concentrated to 9.8 cc., had $\alpha_D^{23} = -1.99°$ (2 dm.), whence $[\alpha]_D = -167°$. Racemization had thus taken place to the extent of 20 per cent. This was borne out by the appearance of the regenerated cystine, which crystallized in imperfectly formed hexagons. The occurrence of so large a proportion of cystine among the products of acid hydrolysis may be explained, not only by the probably complicated nature of the reaction, but by the presence in the original salt of traces of copper, which would facilitate autoxidation.

No attempt was made to apply quantitative methods to the action of alkali, beyond noting the extent to which reducing substances were formed. A solution of 52.1 mg. of the salt in 2 cc. of water, on being treated by the Tompsett procedure, gave no color immediately on the addition of the phosphotungstic reagent, but a blue color developed during the prescribed 8 minute period before dilution. This color became intensified during 20 minutes to a maximum equivalent to that from 1.7 mg. of cystine. In another case 1 cc. of a solution in N NaOH, containing 23.6 mg. of the salt per cc., gave, after standing for 16 hours at room temperature, color equal to that from 4.2 mg. of cystine. A second portion

of this solution, on treatment with barium chloride, gave an appreciable amount of a precipitate identified as barium sulfite. Sulfate was not present in detectable quantity.

Solutions of the salt give no precipitate with copper sulfate, lead acetate, or ferric chloride. Silver nitrate gives none in the cold; but an amorphous white precipitate, soluble in ammonia, separates on boiling. Lead sulfide is formed rather slowly on boiling with dilute alkaline plumbite, more rapidly with alkali of very high concentrations.

Action of Ammonium Sulfite on Cystine

To a solution of 6.00 gm. of cystine in a mixture of 35 cc. of 28 per cent ammonia and 50 cc. of water was added a solution of ammonium sulfite prepared by neutralizing 50 cc. of 28 per cent ammonia with sulfur dioxide. After standing for 48 hours, the cystine which had separated was redissolved by gently warming. The solution was then diluted with water to 250 cc.; it had a rotation of $[\alpha]_{546}^{20} = -108.3°$ (a 4 per cent solution of cystine in 8 per cent ammonia alone showed $[\alpha]_{546}^{20} = -117.6°$). It contained only a trace of sulfate.

On heating a portion with alkaline plumbite for 24 hours at 100°, lead sulfide corresponding to 83 per cent of the sulfur in the cystine was precipitated. This is little, if any, more than that obtainable from cystine without sulfite (2). On the other hand, addition of excess of copper sulfate furnished a dark solution which on acidifying with acetic acid yielded a precipitate resembling the cuprous cysteine complex described above. The filtrate from this precipitate was deep blue. From these observations the existence of an equilibrium between cystine and ammonium sulfite, and their products, may be inferred.

Evaporation of a portion of the solution to dryness on the steam bath led to a grossly decomposed residue containing much free sulfur. On evaporating at room temperature under reduced pressure in a current of air, crystals that resembled impure cystine soon separated. When the volume had been reduced by 20 per cent, the mixture was gently warmed; the crystals redissolved completely and only a small fraction reappeared on standing. This second crop remained permanently in solution after being again warmed. Evaporation as above was continued until crystals of

ammonium sulfite appeared. The mixture was then placed in a vacuum desiccator over concentrated sulfuric acid and soda-lime. When free from liquid, the residue was moistened with water and replaced in the desiccator. After three such treatments, the volatilization of the ammonium sulfite appeared complete; the residue consisted of prismatic needles, which were collected, washed with very small amounts of cold water, and recrystallized.

A further quantity was obtained from the mother liquors by adding small amounts of barium hydroxide, removing barium sulfite, and allowing the filtrate to evaporate. When just sufficient barium hydroxide was employed, the residue on evaporation consisted practically entirely of the same needles. When, however, an excess of barium hydroxide was added and the mixture was boiled to expel ammonia, the only crystalline product obtained on evaporation was barium thiosulfate, identified by its reactions, solubility, crystalline form, and barium content (found 51.5, calculated 51.3; nitrogen was absent).

Ammonium S-Cysteinesulfonate

The product crystallizing in needles displayed general reactions analogous to those of the sodium salt.

Analysis—Total N 12.72, total S 29.38, NH_3-N 6.95, S by Br 15.1

Calculated for $C_3H_{10}O_2NS \cdot SO_3NH_4$. Total N 12.85, total S 29.35, NH_2-N 6.42, S by Br 14.67.

Rotation—$[\alpha]_D^{24} = -104.2°$ (4.14 per cent in water), $-30.5°$ (2.07 per cent in N NaOH), $-59.5°$ (2.07 per cent in N HCl)

It is very readily soluble in water but insoluble in alcohol. Like the sodium salt, it yields sulfate on heating with acids and sulfite with alkalies. The latter reaction takes place quantitatively. A solution of 81.4 mg. of the salt in 4 cc. of N NaOH was allowed to stand at room temperature for an hour and was then treated with 4 cc. of 0.4 N Ba(OH)₂. A bulky precipitate formed at once, and increased in amount on heating to 100°. After an hour at this temperature the precipitate was collected, washed, and covered with 5 cc. of water. After the addition of 1 cc. of concentrated hydrochloric acid below the surface, the mixture was at once titrated with iodine, care being taken to avoid loss of sulfur dioxide. 0.76 milli-equivalent was required, corresponding to 0.38 mM of

barium sulfite (102 per cent of the calculated amount). The resulting barium sulfate weighed 89.9 mg. (0.385 mM, or 103 per cent). The filtrate from the barium sulfite gave only a trace of lead sulfide on treatment with alkaline plumbite in the cold, but rapidly yielded a bulky, black deposit on heating.

SUMMARY AND CONCLUSIONS

In the action of sulfite upon cystine:

1. No sulfate is formed.

2. Only one-half of the cystine is converted to cysteine.

3. The other half is converted into a salt of S-cysteinesulfonic acid.

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THE REACTION OF THE CHICKEN TO IRRADIATED ERGOSTEROL AND IRRADIATED YEAST AS CONTRASTED WITH THE NATURAL VITAMIN D OF FISH LIVER OILS*

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A number of reports have appeared in the literature purporting to show that irradiated preparations such as irradiated ergosterol do not give the same quantitative results per rat unit of vitamin D as cod liver oil, when fed to chickens. Kreitmair and Moll (1) reported that leg weakness in chickens could be cured by the administration of 0.01 mg. of irradiated ergosterol, while toxic effects were not to be feared with the administration of amounts as large as 50 mg. These represent the first results which refer to the therapeutic activity and toxicity of irradiated ergosterol when administered to birds.

Massengale and Nussmeier (2) demonstrated that irradiated ergosterol, equivalent in vitamin D content to 4000 per cent of cod liver oil, greatly

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increased the serum calcium in chickens. However, for the prevention of rickets it required many more vitamin D units from this source than from cod liver oil. A 200 per cent cod liver oil equivalence produced normal bone, but 20 per cent failed to do so.

Hess and Supplee (3) at about the same time reported that many times the required units of vitamin D in cod liver oil failed to protect chickens when given as irradiated ergosterol. They considered this lack of response all the more remarkable because chickens respond so readily to direct treatment with ultra-violet radiations.

Mussehl and Ackerson (4) fed vitamin D to chicks, as irradiated ergosterol equivalent by rat test to 2, 4, 6, 20, 30, and 50 per cent of cod liver oil, and irradiated yeast equivalent to 10 per cent of cod liver oil, with resultant production of rickets.

King and Hall (5) found irradiated ergosterol effective in preventing rickets in chickens, and excessive amounts of it harmful to good bone calcification. They placed no emphasis on discrepancies in quantitative relations.

Taylor, Weld, Branion, and Kay (6) found chickens on a diet of corn, wheat, and oats remarkably resistant to large doses of irradiated ergosterol. With doses which they considered 25,000 times the therapeutic size no harmful effects resulted and even the serum calcium was not increased. Their birds, however, were kept on a diet of corn, wheat, and oats, and therefore were possibly on a low calcium ration.

In our present experiments we sought first of all to verify the experiences of other investigators, hoping at the same time to put relations on a more quantitative basis, and then, if possible, ascertain the reasons for the difference in effectiveness of the natural vitamin D of cod liver oil as compared with the irradiated product. We surmised a difference in the character of the active compounds or as an alternative that other substances present in cod liver oil might increase the efficiency with which its vitamin D functioned. Incidentally we also included a few experiments on irradiated molds and one on commercial egg meal.

<div align="center">EXPERIMENTAL</div>

In our first experiments we ran individual series, using baby chicks 2 to 3 days after hatching. For the most part they were white Leghorns, but in Series I, IV, and V we used Plymouth Rocks. They were provided with brooders and a small runway with wood shavings as litter. Fifteen to twenty birds were fed in each group.

As a ration there was given skim milk as a drink—instead of water—with a dry mash of yellow corn 97, calcium carbonate 2,

and sodium chloride 1. Except for Series I, the calcium as carbonate was furnished entirely as precipitated $CaCO_3$. In Series I, the calcium was furnished in the form of 1 part of pearl grits ($CaCO_3$) and 1 part of raw bone per 100 parts of dry mash. The ergosterol was incorporated in the ration in the form of a corn oil solution which was diluted with ether to effect distribution. New rations were mixed at 7 to 10 day intervals. Care was taken to secure complete consumption of contents of food containers to eliminate selection. The yeast fed was finely ground Fleischmann's yeast irradiated in the dry condition to an antirachitic potency of 0.5 Steenbock rat unit per mg. It required approximately 0.001 mg. of irradiated ergosterol to furnish this activity. The cod liver oil used was of standard potency containing 13.3 rat units per gm.[1]

Series I, II, III, and VI were run for 7 weeks and the others for 8. In general we have found a great difference in the activity and vitality of different hatches probably due, not only to their management, but also to heredity and storage reserves. These variations made us change our methods of selecting material for analysis. In Series I, II, III, and VI we eliminated a few individuals which were obviously abnormal due to causes other than rickets and then selected the ten poorest. In Series IV and V we eliminated the obviously abnormal ones and then selected the four heaviest and the four lightest. Obviously with such variations the data of different series are not strictly comparable with one another. Another factor which merits consideration in this connection is the technique of feeding liquid milk separately. With variation in the amount of milk consumed in consequence of change of temperature and humidity of the air, and also activity of the birds, the rickets-producing tendency of the diet undoubtedly was not the same in all the series. We were aware of this difficulty but at the time that these experiments were performed the ration used by us had given more satisfactory results than any other. As

[1] As 1 rat unit we have designated the total amount of vitamin D which will produce a narrow line of calcium deposit in the rachitic metaphyses of the distal ends of the radii and ulnæ of standard rachitic rats in a period of 10 days. This represents the unit which was later accepted for "New and non-official remedies" by the American Medical Association. It is approximately equivalent to 2.7 international units.

criteria for differences in protective value we used ash analyses of dried and alcohol-extracted tibiæ.

The results from the first series of experiments are shown in Table I.   In Series I and II it is revealed that it required 40 to 120 times more units of vitamin D in the form of irradiated ergosterol to effect calcification equal to that obtained with cod liver oil. And in the other series yeast also showed itself to be a poor source of vitamin D for this species.   In Series I 0.4 per cent of yeast was equal in calcifying activity to 0.2 per cent of cod liver oil; in Series III, IV, and V 1.0 to 1.6 per cent was as effective as 2 per cent of cod liver oil; and in Series VI 0.2 per cent was as effective as 1 per cent of cod liver oil.

It is obvious that no exact conclusions regarding the comparative efficiency of yeast, ergosterol, and cod liver oil can be drawn because the variations in different series are too large, but if anything, they demonstrate the inefficiency with which both irradiated ergosterol and yeast serve as antirachitic agents for the chick. Evidently with a slight variation in conditions their effectiveness for producing normal bone is greatly modified.   If our basal ration had been productive of a less severe rickets, and if it had been more satisfactory otherwise, the chances are that the results of the different series would have been more concordant.

With the marked difference in antirachitic value of vitamin D from cod liver oil, yeast, and ergosterol demonstrated, it becomes important to know if other natural sources of vitamin D are generally as efficient as cod liver oil.   With this in mind we determined the value of burbot liver oil[2] in series with the latter. That burbot liver oil contains vitamin D has already been shown by McCollum and Simmonds (7), Clow and Marlatt (8), and Branion (9).

Thirty-six white Leghorn baby chicks were divided into three equal groups.   One was put on a rickets-producing basal ration, one on the same plus 1 per cent of cod liver oil, and the third on the basal plus 1 per cent of burbot liver oil.   The basal ration used was the ration described by Hart, Kline, and Keenan (10). It consisted of yellow corn 59, wheat middlings 25, crude casein 12, sodium chloride 1, calcium carbonate 1, tricalcium phosphate

[2] We are indebted to Smith Brothers of Port Washington, Wisconsin, for the burbot liver oil.

TABLE I

Antirachitic Efficiency of Irradiated Ergosterol and Yeast for the Chicken

Series No.	Ration supplement	Average final weight	Weight of tibia	Tibia ash
		gm.	*gm.*	*per cent*
I	None	234	1.04	31.0
	0.2 per cent c. l. o.	255	1.15	34.1
	1.0 " " "	326	1.34	42.2
	20.0 " " " equivalent as ergosterol	322	1.36	35.8
	40.0 " " " " " "	383	1.51	38.2
	120.0 " " " " " "	378	1.38	40.4
	0.4 " " irradiated yeast (15 per cent c. l. o.)	348	1.23	34.5
II	2.0 per cent c. l. o.	207	0.55	45.8
	4.0 " " " equivalent as ergosterol	179	0.52	39.6
	40.0 " " " " " "	208	0.62	45.1
	360.0 " " " " " "	184	0.55	47.1
III	None	158	0.53	34.7
	0.2 per cent irradiated yeast (7.5 per cent c. l. o.)	267	0.82	42.5
	1.0 " " " " (37.5 " " ")	262	0.78	46.3
	2.0 " " c. l. o.	256	0.74	47.8
IV	None	196	0.78	38.1
	2.0 per cent c. l. o.	391	1.55	48.6
	0.4 " " irradiated yeast (15.0 per cent c. l. o.)	389	1.68	41.1
	1.6 " " " " (60.0 " " ")	485	1.70	48.4
	1.6 " " non-irradiated yeast	226	1.08	35.8
V	None	274	1.30	38.1
	2.0 per cent c. l. o.	352	1.17	46.9
	0.4 " " irradiated yeast (15.0 per cent c. l. o.)	396	1.24	44.9
	1.6 " " " " (60.0 " " ")	431	1.39	45.9
	6.4 " " " " (240.0 " " ")	461	1.65	48.0
	6.4 " " non-irradiated yeast	430	1.64	41.3
	1.6 " " irradiated *Penicillium*	404	1.35	44.5
	4.0 " " egg meal	366	1.33	42.3
VI	None	217	0.67	35.6
	1.0 per cent c. l. o.	234	0.74	45.8
	0.1 " " irradiated yeast (3.75 per cent c. l. o.)	261	0.76	43.2
	0.2 " " " " (7.50 " " ")	287	0.36	46.2
	0.5 " " " " (18.75 " " ")	257	0.73	46.0
	1.5 " " " " (56.25 " " ")	268	0.76	45.8

1, and dried yeast 1, with water to drink. Both the cod liver oil and the burbot liver oil were of standard potency; *i.e.*, 75 mg. contained 1 Steenbock rat unit. The chicks were kept on screen bottoms. They were weighed weekly and at the end of 5 weeks the ten best chicks of each group were killed and their tibiæ analyzed for ash. Percentages were calculated on the alcohol-extracted dry basis. The results from this experiment are shown in Table II. It is apparent that burbot liver oil is as efficient per rat unit of vitamin D as cod liver oil.

Judging from the reduced effectiveness of ergosterol as compared with cod liver oil and burbot liver oil, one is forced to surmise, either that the vitamin D produced by irradiation is a different substance from that found in cod liver oil, or that cod liver oil

TABLE II

Antirachitic Efficiency of Burbot Liver Oil

	Final average weight	Ash of tibiæ	
	gm.	*gm.*	*per cent*
Basal ration......................	131	0.46	33.2
" " + 1 per cent c. l. o...	302	0.96	48.6
" " + 1 " " burbot liver oil.......................	331	1.10	49.1

C. l. o. = cod liver oil.

contains something which has an effect upon the efficiency with which vitamin D functions. In order to determine which of these two states of affairs could be accepted as true to fact, we determined whether irradiated ergosterol dissolved in cod liver oil had a greater calcifying effect than when it was dissolved in a neutral oil such as corn oil.

It is a well known fact that in the hen, as well as in other birds, there results an increase in blood calcium during egg laying to a degree which would be considered unphysiological in mammals, and which can be produced in them only by the administration of exceedingly large amounts of irradiated ergosterol or parathormone. It was, therefore, to be expected that the tolerance of the bird for irradiated ergosterol would be much higher than that of the mammal. In spite of this, we decided that it might be ad-

vantageous, in our first experiments, to use the technique of determining whether or not cod liver oil had the ability to increase the toxicity of irradiated ergosterol. We preferred this technique to a determination of comparative antirachitic activities because of the greater rapidity with which results could be obtained and because of the greater amount of cod liver oil which could be added.

We ran these experiments in series. In the first we used eleven birds which had received previously 0.2 per cent of irradiated yeast in a rachitogenic diet of yellow corn 97, calcium carbonate 2, sodium chloride 1, and skim milk *ad libitum*. These birds had been on their ration for 7 weeks and were entirely normal in external appearance, although analysis of the bones of individuals from the group showed a rachitic state. They were transferred to the Wisconsin chick ration (11) which consists of 80 pounds of yellow corn, 20 pounds of wheat middlings, 5 pounds of raw bone, 5 pounds of calcium carbonate grit, 1 pound of sodium chloride, and skim milk to drink. Four birds were given, in addition, 2 per cent of cod liver oil carrying in solution 532,000 rat units of vitamin D as irradiated ergosterol. Four other birds were given corn oil instead of cod liver oil carrying the same amount of irradiated ergosterol, and three birds were kept as controls. They were run in two series, two birds on each of the oils, with two birds kept as controls in one case and one bird in the other. The ergosterol used was furnished by The Fleischmann Company and was irradiated dissolved in ether in our laboratory. After irradiation the ether was distilled off and the product dissolved in the oils. The oils were incorporated directly in the ration at frequent intervals.

Inasmuch as we have defined a standard cod liver oil as one containing 13.3 rat units per gm., the administration of 532,000 rat units of vitamin D as irradiated ergosterol represented the equivalence of approximately 40,000 per cent of cod liver oil. This, of course, is an enormous dosage. The cod liver oil which we used was a commercial product which we found, by repeated assay with rats, to approximate in potency that designated by us as standard. Analysis for vitamin A by the ophthalmic method as used with rats revealed that 3 mg. of the oil daily were able to cure a severe ophthalmia in the course of 10 days. The oil was of

a light yellow appearance and we had every reason to believe that it represented an average commercial product.

The birds were kept in individual cages and the consumption of the respective dry mashes was recorded daily. No record was made of the skim milk consumed. The control birds started with a consumption of 20 gm. daily, which gradually increased to 50 gm. The ergosterol-fed animals, however, all dropped off in consumption by the 5th day and some showed this effect even on the 3rd day. Towards the end of the 7 day period, they consumed only a few gm. daily. The birds were weighed daily and showed changes in weight which correspond very closely to their feed consumption. The control birds gained consistently while those receiving the irradiated ergosterol began to lose weight beginning with the 5th day. Blood analyses were made by the Fiske-Subbarow (12) method for phosphorus and the Clark-Collip (13) method for calcium. The samples were taken at the end of the 7 day period when the birds were killed. The tibæ were dissected out, extracted with alcohol in a Soxhlet extractor for a few days after crushing, then dried and ashed. The kidneys, liver, heart, spleen, and thymus were removed and dried in a steam oven at 100°. The kidney and heart samples, after drying, were ashed and analyzed for calcium by a microanalytical method based upon the Clark-Collip procedure.

The results of the experiments of the first series are shown collectively in Tables III and IV. The final weights of the birds receiving irradiated ergosterol were less than the controls in both instances. There was no marked variation in the dry weights of the tibiæ. But the liver, lungs, heart, and spleen of the ergosterol-fed animals were lighter. The thymus, however, which has been reported to undergo marked atrophy in mammals during hypervitaminosis showed no consistent change. As the amount of vitamin D which was consumed in corn oil and cod liver oil solution was approximately the same in both cases, the uniformity of effect indicates that cod liver oil did not increase its toxicity.

Table IV which deals with the variation in tissue composition reveals no change in the ash of the tibiæ whether calculated on a weight or percentage basis. Serum calcium was increased with both solvents approximately twofold. With this, the inorganic phosphorus in the serum was correspondingly reduced. The

kidney calcium was increased, on the average from 7 to 11 times over the normal, and the heart calcium was in some cases doubled.

It was very evident that in these experiments the dosage with vitamin D was excessive. It was probable that smaller dosages would have been more effective in bringing out possible differences. For this reason, another series of experiments was started in which the dosage was reduced from 532,000 to respectively 100,000 and 250,000 rat units of vitamin D per 100 gm. of ration. The prepara-

TABLE III

Effect of Excessive Ergosterol Dosage in Corn Oil and Cod Liver Oil on Weight Relations in 7 Day Experiments

Chick No.	Ration modification	Vitamin units consumed	Initial weight	Final weight	Dry weight of organs					
					Tibia	Kidneys	Liver	Heart	Spleen	Thymus
		thou-sands	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
3470	None	0	400	466	1.63	1.01	3.50	0.70	0.13	
3524		0	524	580	1.86	0.85	4.04	0.92	0.21	0.37
3713		0	500	595	1.68	1.10	3.89	0.73	0.21	
3515	532,000 vitamin D units	265	475	430	1.45	1.42	2.63	0.48	0.13	
3623	in 2 gm. *corn oil* per 100	592	537	472	1.80	1.52	3.25	0.59	0.15	
3686	gm. ration (40,000 per	520	590	590	1.90	1.51	3.45	0.75		0.30
3695	cent c. l. o. equivalent)	395	499	440	1.70	1.11	2.89	0.49	0.17	0.25
3461	Same amount of vitamin	301	420	297	1.28	1.10	2.39	0.31	0.09	
3506	D in 2 gm. *c.l.o.* per 100	421	475	379	1.54	1.39	2.86	0.45	0.12	
3497	gm. ration	410	524	449	1.62	1.36	3.20	0.52	0.15	0.39
3650		546	469	385	1.57	1.04	3.76	0.54	0.10	0.14

C. l. o. = cod liver oil.

tions used were the same as those fed in the previous experiments. Three birds were kept as controls and three were put on each of the levels of corn oil and cod liver oil, making fifteen birds in all. The birds were white Leghorns raised on the Wisconsin ration plus skim milk *ad libitum,* from the time of hatching to an age of 46 days, when the irradiated ergosterol was incorporated in the ration. The experiment was continued for 32 days except in the instances when the birds died before the expiration of this time. This

happened with four birds receiving 250,000 units of vitamin D, after they had lost considerably in weight; however, it also occurred in one of the controls and in two birds receiving 100,000 units of vitamin D. In these, death was not preceded by a loss in weight. We were unable to determine the cause of death in these instances. The dry mash consumed and the weights of the birds were recorded daily. The skim milk consumed was not recorded.

TABLE IV

Effect of Excessive Ergosterol Dosage in Corn Oil and Cod Liver Oil on Tissue Composition in 7 Day Experiments

Chick No.	Ration modification	Tibia ash		Per 100 cc. serum		Per 100 gm. dry tissue	
				Calcium	Phosphorus	Kidney Ca	Heart Ca
		gm.	*per cent*	*mg.*	*mg.*	*mg.*	*mg.*
3470	None	0.62	38.6*	8.3	7.7	39	26
3524		0.90	43.3			39	26
3713		0.78	46.5	10.6	6.8	61	27
3515	532,000 vitamin D units	0.71	49.1	17.1	3.7	149	43
3623	in 2 gm. *corn oil* per 100	0.84	47.7	18.9	5.2	444	58
3686	gm. ration (40,000 per	0.91	48.0			341	39
3695	cent c. l. o. equivalent)	0.80	47.4			404	52
3461	Same amount of vitamin	0.58	45.5	18.2	4.1	809	67
3506	D in 2 gm. *c. l. o.* per 100	0.72	46.1	18.8	3.9	488	52
3497	gm. ration	0.79	49.1			372	23
3650		0.75	47.8			415	50

C. l. o. = cod liver oil.
* Killed at the beginning of the experiment.

The results obtained were similar to those already described. In detail they are presented in Tables V and VI. At an intake of 250,000 units of vitamin D, there was a pronounced loss in body weights as compared with the initial weights. At 100,000 units the loss was not so consistent, but in both instances, the losses were not any greater on cod liver oil than on corn oil. In relation to the dry weights of the organs, the weights of the kidney, liver, heart, and spleen were noticeably lower with the higher levels of

intake. However, except in the case of the spleen, the lowered weights may be explained by the lesser body weight of the animals at the time of termination of the experiments.

TABLE V

Effect of Excessive Ergosterol Dosage in Corn Oil and Cod Liver Oil on Weight Relations in 21 to 32 Day Experiments

Chick No.	Ration modification	Vitamin units consumed	Duration of experiment	Initial weight	Final weight	Dry weight of organs				
						Kidneys	Liver	Heart	Spleen	Lung
		thousands	days	gm.	gm.	gm.	gm.	gm.	gm.	gm.
8901	None	0	32	360	750	1.26	4.42	0.78	0.37	0.88
8933		0	32	290	480	0.95	3.00	0.50	0.20	0.55
8937		0	26*	285	370	1.11	3.71	0.63		0.61
8842	100,000 vitamin D units	447	32	365	415	1.42	2.40	0.42	0.08	0.43
8911	in 2 gm. *corn oil* per 100	150	24*	310	150	1.21	1.79	0.26	0.02	0.36
8935	gm. ration (7500 per cent c. l. o. equivalent)	492	32	270	385	1.24	2.64	0.40	0.09	0.45
8862	100,000 vitamin D units	268	31*	305	320	2.05	3.40	0.31		0.46
8907	in 2 gm. *c. l. o.* per 100	410	32	275	320	1.46	2.43	0.29	0.08	0.39
8927	gm. ration	367	32	365	310	1.58	2.15	0.30	0.06	0.41
8856	250,000 vitamin D units	417	21*	340	204	0.94	1.39	0.37	0.04	0.42
8909	in 2 gm. *corn oil* per 100	322	25*	345	250	1.34	2.35	0.32	0.02	0.35
8934	gm. ration (18,800 per cent c.l.o. equivalent)	622	32	255	240	0.54	1.23	0.22	0.04	0.21
8890	250,000 vitamin D units	290	24*	320	168	1.08	1.19	0.20		0.54
8924	in 2 gm. *c. l. o.* per 100	302	26*	350	175	0.78	1.04	0.20		0.36
8930	gm. ration	397	32	280	260	1.03	2.77	0.33	0.04	0.25

C. l. o. = cod liver oil.
* Bird found dead in cage.

Table VI shows that the percentage of ash in the tibiæ underwent no characteristic change; however, the serum calcium was markedly increased with the corresponding decrease in the serum phosphorus. In all cases where vitamin D had been administered, there were no indications that cod liver oil increased the

changes in tissue composition.   Analyses of the kidneys and heart tissues showed an absence of effect of cod-liver oil as compared with corn oil, but in all cases with the administration of irradiated ergosterol a pronounced increase in calcium occurred.   This was most pronounced in the kidneys.

TABLE VI

Effect of Excessive Ergosterol Dosage in Corn Oil and Cod Liver Oil on Tissue Composition in 21 to 32 Day Experiments

Chick No.	Ration modification	Tibia ash		Per 100 cc. serum		Per 100 gm. dry tissue	
				Cal-cium	Phos-phorus	Kidney Ca	Heart Ca
		gm.	*per cent*	*mg.*	*mg.*	*mg.*	*mg.*
8901	None	1.10	44.6	11.7	8.4	84	31
8933		0.70	46.8	10.9	5.9	60	54
8937		0.54	45.8			151	45
8842	100,000 vitamin D units	0.77	47.7	14.9	4.7	410	56
8911	in 2 gm. *corn oil* per 100	0.72	47.3			1315	71
8935	gm. ration (7500 per	0.67	46.7	16.3	4.6	330	118
	cent c. l. o. equivalent)						
8862	100,000 vitamin D units	0.64	46.9			838	65
8907	in 2 gm. *c. l. o.* per 100	0.75	48.8	16.3	5.0	470	63
8927	gm. ration	0.60	45.5	16.7	4.2	626	69
8856	250,000 vitamin D units					558	72
8909	in 2 gm. *corn oil* per 100	0.71	47.1			277	119
8934	gm. ration (18,800 per	0.30	46.4	17.5	4.6	431	114
	cent c. l. o. equivalent)						
8890	250,000 vitamin D units	0.51	45.5			579	
8924	in 2 gm. *c. l. o.* per 100	0.46	46.6			942	147
8930	gm. ration	0.48	50.2	14.5	5.9	556	100

C. l. o. = cod liver oil.

We finally carried out some experiments in which we determined the *curative* effect of ergosterol when given with small amounts of cod liver oil.   The ergosterol was so adjusted that it was exactly equivalent in vitamin D units to 1 per cent of cod liver oil as determined by rat assay.   This was fed with two levels of cod liver oil,

namely, 0.05 and 0.10 per cent. While these amounts were known to exercise little or practically no effect upon calcification themselves, it was, however, thought possible that they might contain a sufficient quantity of an unknown substance capable of augmenting the therapeutic effect of the ergosterol. As controls, there were included one group of animals which received the basal ration only and one which received 1 per cent of cod liver oil.

Fifteen white Leghorn chicks were used for each group. They were started shortly after hatching and continued on their rations for 8 weeks. The basal ration consisted of yellow corn 97, calcium carbonate 2, sodium chloride 1, with skim milk to drink *ad libitum*. The cod liver oil and irradiated ergosterol were the same as those used in previous experiments. Ash analyses on the tibiæ were run in the usual manner.

TABLE VII

Effect of Ergosterol Administered with Small Doses of Cod Liver Oil

Ration supplement	Ash in tibiæ
	per cent
1. No supplement..	37.8
2. 1 per cent cod liver oil.................................	46.9
3. Irradiated ergosterol, ≈ 1 per cent cod liver oil.......	37.5
4. Same as (3) + 0.05 per cent cod liver oil..............	37.7
5. " " (3) + 0.10 " " " " "	38.6

From Table VII it is seen that irradiated ergosterol fed at such a level that in vitamin D units the intake is equivalent to 1 per cent of cod liver oil had absolutely no effect upon calcification. 1 per cent of cod liver oil, however, was very effective, resulting in the production of normal bone. When the ergosterol was fed with cod liver oil, calcification was not increased, except possibly in the instance where the cod liver oil was fed at a level of 0.10 per cent. In this case the effect undoubtedly was due to the cod liver oil itself and not to the ergosterol.

Since it has been shown that carotene can prevent the symptoms of vitamin A deficiency in the rat, as it is converted into vitamin A (14, 15), we availed ourselves of its use in further studies of possible factors which may influence the action of vitamin D. For this purpose we used a petroleum ether extract of yellow carrots,

which was administered in corn oil solution. Each bird was given daily 50 times the daily amount necessary to cure an erythemic ophthalmia in a rat in 10 days. In terms of carotene this was equivalent to about 0.050 mg. daily. As experimental birds we used thirteen white Leghorn chicks which had received vitamin D as irradiated ergosterol equivalent to 5 per cent of cod liver oil, in addition to yellow corn, sodium chloride, and skim milk for 7 weeks. The seven best birds were continued on this ration; the others were given the carotene supplement. After 45 days they were killed and their tibiæ analyzed for ash in the usual manner.

From the results shown in Table VIII it is apparent that carotene was without effect. This duplicates the experience of Hunter and Knandel (16) who used alfalfa powder as the source of carotene and of Russell and Klein (17) who found that dried carrots had no

TABLE VIII

Effect of Carotene upon Antirachitic Efficiency of Irradiated Ergosterol

	Weight of birds		Ash in tibiæ	
	Initial	Final		
	gm.	*gm.*	*gm.*	*per cent*
Without carotene..................	294	616	0.86	42.2
With "	272	544	0.84	42.8

effect on the efficiency of action of irradiated ergosterol in chickens. It is, of course, possible that the chick may differ from the rat in its ability to transform carotene into vitamin A, but Mr. Baumann of this laboratory (18) has shown that the administration of carotene to the laying hen results in an increase in the vitamin A content of the egg yolk. This occurs without an increase in pigmentation. Evidently the cause of the lesser efficiency of the vitamin D of ergosterol is not to be sought in vitamin A relations.

SUMMARY

Ergosterol and yeast when activated antirachitically by ultra-violet radiations are inefficient sources of vitamin D for the chicken. Whereas 1 per cent of cod liver oil of average potency resulted in normal bone production, it required from 40 to 120 per cent cod liver oil equivalence as irradiated ergosterol and from 7.5 to 60

per cent cod liver oil equivalence as yeast to produce the same result. With yeast this represented an actual consumption of 0.2 and 1.6 per cent of the weight of the dry mash. Good results were also obtained with 4 per cent of egg meal and 1.6 per cent of an irradiated *Penicillium*.

Burbot liver oil revealed the same antirachitic efficiency for chickens as cod liver oil, when compared on the basis of rat units of vitamin D contained therein.

Irradiated ergosterol in excessive doses was found to be toxic for chickens. This toxicity was manifested by anorexia, loss in body weight, and a loss in dry organ weight of spleen, heart, liver, and lung. The blood serum calcium was increased and the phosphorus was decreased. With the higher levels of intake the heart calcium was frequently doubled and the kidney calcium was increased 10 times and more.

The vitamin D produced by ordinary irradiation of ergosterol with a quartz mercury vapor lamp is a different substance from that found in cod liver oil. This is concluded from the fact that irradiated ergosterol whether fed in corn oil or in cod liver oil solution shows the same degree of antirachitic efficiency and the same toxicity.

The administration of carotene had no effect on the antirachitic activity of irradiated ergosterol.

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VITAMIN D AND THE CONSERVATION OF CALCIUM IN THE ADULT. I*

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The general importance of the conservation of calcium and phosphorus in the adult organism makes itself evident in many regions, both among peoples and animals. In the orient, for example, there frequently is observed a malady known as osteomalacia. It is a case of impaired metabolism of calcium and phosphorus caused by an inadequate diet, a lack of a proper hygienic environment, or both. As a physiological contrast, we have the accelerated metabolism of these elements in the lactating dairy cow and the laying fowl.

For obvious reasons the rat has served frequently in various studies of calcium and phosphorus metabolism. Accordingly, different dietaries have been constructed from time to time to provide a supply of normal as well as uniform animals. Some of these diets have been rather simple in composition. Thus it has been claimed by Sherman and his collaborators (1) that with the rat good growth and skeletal development and reproduction may be obtained on a diet constructed from ground whole wheat, whole milk powder, and sodium chloride. Though our experience with this diet has not demonstrated normal performance, we have, on the whole, been impressed with the reproduction obtained.

That the rat is normally resistant to rickets is revealed by the fact that the newly weaned rat with its rapid rate of growth can be rendered rachitic only after having been fed a diet exceedingly

low in calcium or one in which the calcium and phosphorus ratio is seriously disturbed. In the adult, with growth removed as a contending factor, there needs to be met only the requirements for maintenance of the soft tissues and the abundant skeletal deposits of calcium and phosphorus except as these may be increased by pregnancy and lactation. With this in mind, one may well raise the question, Does the adult rat require vitamin D for its normal calcium metabolism and conservation under certain circumstances, or does it benefit at all by the inclusion of vitamin D in its diet?

Three methods of approach to the solution of this question appear open. One is to make the conditions unfavorable for the conservation of calcium and phosphorus by using a rickets-producing diet carrying an unfavorable ratio of calcium to phosphorus. Another is to feed a low calcium diet, and a third is to use a diet accepted as normal, but to impose upon the animal the strain of lactation which is well known to be unfavorable for the conservation of calcium and phosphorus reserve.

We have availed ourselves of all three of these methods, but shall report on only two at the present time. In one we used our rickets-producing Ration 2965 (2) with and without vitamin D for maintenance of adult male and female rats; in the other, we used our stock diet (3) slightly modified, with and without vitamin D during periods of reproduction and lactation.

<div style="text-align:center">EXPERIMENTAL</div>

Male Maintenance Series—In this series, we compared the effect of Ration 2965 as such, and as supplemented with vitamin D by irradiation, on the conservation of body calcium and phosphorus by the rat over a period of 205 days. The comparison was made on the basis of the ash content of their femurs and the calcium and inorganic phosphorus content of the blood. Inasmuch as Sherman and Hessler (4) have shown that the amount of calcium present in the femurs of rats may be used to calculate the approximate total amount of calcium present in the body of the rat, and inasmuch as the percentage of calcium and phosphorus in bone ash is fairly constant, we accepted the determination of femur ash as a convenient method for measuring calcium reserves.

For controls we used rats maintained on our modified stock diet. This consisted of our usual stock diet in which the grain

mixture was altered so as to have the following composition: 71.5 yellow corn, 15.0 oil meal, 5.0 casein, 2.0 alfalfa, 1.0 bone ash, 5.0 butter fat, 0.5 NaCl, and the raw whole milk was replaced by whole milk powder. The latter was mixed with the grain portion in an amount equal to one-third of the final weight.

To supply vitamin D when wanted, Ration 2965 was irradiated. This was done as follows: 200 gm. were evenly distributed in each of four galvanized pans 2 feet square and then exposed to the radiations of a Cooper Hewitt or Luxor quartz mercury vapor lamp for a total of 30 minutes at a distance of 2 feet. After 15 minutes of exposure the product was stirred thoroughly and then irradiated to completion. This process of irradiation gave a degree of potency in vitamin D not exceeding 1 Steenbock rat unit per 20 gm. of ration. Parenthetically, it may be stated that 1 rat unit is that amount of vitamin D which in 10 days will produce a narrow continuous line of calcium deposits in the distal ends of radii and ulnæ of standard rachitic rats kept on Ration 2965 (5).

Thirty-six animals, 175 days old as selected from our stock colony, were used for the experiments. They ranged in weight from 295 to 415 gm. They were divided with respect to weight into four groups of equal number. One group was killed at the initiation of the experiment to serve as initial controls, one was placed on Ration 2965, one on Ration 2965 irradiated, and one on the modified stock diet to serve as a final control. They were kept on screens, two animals to a cage. They were weighed once a week. Consumption of food was *ad libitum*.

When taken for analysis, the animals were placed under light ether anesthesia and bled from the carotid artery. The serum which separated from the whole blood, on standing at ice box temperature for 24 hours was analyzed for inorganic phosphorus according to the Fiske-Subbarow method (6) and for calcium according to the Clark-Collip (7) modification of the Kramer-Tisdall method (8). The femurs were removed, dissected free from soft tissues, and extracted in a Soxhlet extractor with frequent changes of fresh alcohol for a total of 5 days. They were then dried in an oven at 100° and ashed in a muffle furnace. The ash was calculated in terms of per cent of the weight of the dry extracted bones. The data summarized in Tables I and II will be discussed later with the data from the female maintenance series which follow.

Female Maintenance Series—The work in this series was a duplication of the male maintenance series with two exceptions. In the first place the food consumption was equalized by limiting the consumption of all animals to the smallest amount voluntarily consumed by any animal not obviously abnormal. In the second place, the use of a control group running concurrently on the modified stock diet was omitted. The animals were 175 to 190 days old and ranged in weight from 212 to 260 gm. They were equally divided with respect to weight into three groups. Twenty-seven animals were used. One group was killed at the initiation of the experiment as before; one was fed the irradiated diet, and one the non-irradiated. The experimental diets were fed over a period of 202 days with the animals segregated in individual cages. In the taking of blood samples the blood of two or three animals was pooled for analysis. Later, females comparable in age to those on experiment were taken from the stock colony for additional blood analyses. The resulting data together with those of the preceding series are presented in Tables I and II.

On analyzing the data in Table I it will be seen that the average percentage of ash in the femurs of males and females on Ration 2965 is very much lower than in the respective controls taken at the initiation of the experiment. More conspicuous are the differences in the actual average weights of femurs and femur ash of the two respective groups. If the average femur weight of the corresponding initial controls is given a value of 100, in each case, that is for males and females respectively, the average femur weight of the males can be represented by 77 and the average femur weight of the females by 82. This decrease in weight is reduced but not entirely compensated for by the feeding of Ration 2965, irradiated. In this instance, the males and females gave respective values of 91 and 99. Males continued concurrently on the modified stock diet showed a slight increase in average femur weight over the average femur weight of the initial controls. Females, it will be recalled, were not included in such tests.

What has been shown with respect to the average femur weights also applies to the values of average femur ash in the dry extracted bones. If the average femur ash of the initial controls is given a value of 100, the average femur ash weight of males and females on Ration 2965 can be represented by 66 and 71, respectively. With

the irradiated Ration 2965 the average femur ash weight was increased to 88 with the males, and to 93 with the females.

On analyzing the picture of the calcium and inorganic phosphorus of the bloods as presented in Table II it is observed that the values for calcium fall within the usual limits; but this is not the case with respect to inorganic phosphorus. Whereas the males on the stock diet as such or as modified show an average of

TABLE I

Analyses of Femurs of Adult Rats on Rachitogenic and Non-Rachitogenic Diets

Ration	Range in weight of femurs	Range in weight of ash	Range of ash
Males			
	gm.	*gm.*	*per cent*
2965	0.410–0.566	0.208–0.307	50.8–58.5
	(0.474)	(0.264)	(55.7)
2965, irradiated	0.471–0.675	0.284–0.429	59.7–65.9
	(0.558)	(0.352)	(63.0)
Stock diet, initial	0.548–0.677	0.351–0.435	63.3–66.1
	(0.613)	(0.398)	(64.9)
" " final	0.591–0.745	0.380–0.492	63.3–66.6
	(0.642)	(0.418)	(65.1)
Females			
2965	0.321–0.400	0.174–0.237	52.0–61.9
	(0.364)	(0.213)	(58.3)
2965, irradiated	0.371–0.482	0.224–0.310	60.3–67.0
	(0.439)	(0.280)	(63.7)
Stock diet, initial	0.385–0.509	0.255–0.346	65.7–68.4
	(0.444)	(0.299)	(67.3)

The figures in parentheses are averages.

6.5 mg. of inorganic phosphorus per 100 cc. of serum, the males on Ration 2965 have only 2.1 mg. while those on the irradiated diet have 4.1 mg. In the case of the females similar results are revealed. However, the inorganic serum phosphorus values of the animals on the irradiated diet more nearly approximate the values for animals on the stock diet.

The weight records which have not been included in Tables I

and II showed that the males on Ration 2965 and Ration 2965 irradiated showed an average loss in weight of 7 and 6 per cent, respectively, from their initial experimental weights. In the case of the females there was an average loss of 5 per cent in the initial experimental weights of the two groups. Considering the quality of the protein of the ration these losses are not large. It may be suggested that some deficiency in vitamin A may have been encountered with the use of our Ration 2965 because it is well known

TABLE II

Effect of Rachitogenic and Non-Rachitogenic Diets on Serum Calcium and Serum Inorganic Phosphorus of Adult Rats

	Ca per 100 cc. serum				P per 100 cc. serum			
	Ration 2965	Ration 2965, irradiated	Stock ration, initial	Stock ration, final	Ration 2965	Ration 2965, irradiated	Stock ration, initial	Stock ration, final
	mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
Males, individual samples	9.6	9.3	9.4	9.3	1.8	3.2	6.2	6.0
	9.7	10.1	9.8	9.5	1.9	3.4	6.4	6.1
	10.0	11.0	9.9	9.7	2.1	3.4	6.4	6.2
	10.5	11.3	10.4	9.8	2.1	4.6	7.2	6.7
	10.5	11.4		10.1	2.2	4.6		6.8
	10.8	11.4			2.6	4.9		7.0
						5.0		7.0
Average..........	10.2	10.7	9.9	9.7	2.1	4.1	6.5	6.5
Females, pooled samples of 2 and 3 animals	9.1	9.7			1.9	5.1		5.3
	9.2	9.8			1.9	5.1		5.3
	9.2	10.0			1.8	5.2		5.7
Average..........	9.2	9.8			1.9	5.1		5.4

that yellow corn does not provide an abundance of vitamin A in comparison with the rat's requirements. However, in these animals the eyes were bright and beaded and the vaginal smears, with the exception of one animal, showed no persistence of cornified cells.

We conclude from the results presented above that when adult rats, either male or female, are placed for long periods of time on a rachitogenic diet such as Steenbock and Black's Ration 2965, minimal amounts of vitamin D such as are formed by irradiation

of the ration are effective in curbing but not entirely preventing losses of skeletal calcium and phosphorus.

Pregnancy and Lactation Series—In this series, we have determined among other things, the influence of vitamin D in conserving the skeletal calcium and phosphorus of rats subjected to pregnancy and lactation on a diet apparently adequate for maintenance as well as for ordinary reproduction. We have also determined the influence of vitamin D fed to females on the calcium content of their young. In view of the calcium storage observed in females during pregnancy by Goss and Schmidt (9) which they could not account for by the calcium present in the young and adnexa at birth, we were led to determine, in this respect, the effect of two successive pregnancies uncomplicated by lactation.

The basal diet used was a modification of our stock diet as given above. Instead of using whole milk powder as before, we substituted skim milk powder and butter fat in the proportion of 72 of the former to 28 of the latter. Furthermore, to supply additional vitamin B for lactation needs we added 3 per cent of dried brewers' yeast. The actual composition of the basal diet used was as follows: 191 parts of grain mixture of our modified stock diet, 9 parts of dried yeast, 72 parts of skim milk powder, 28 parts of butter fat. This diet contained 665 mg. of calcium and 745 mg. of phosphorus per 100 gm. The calcium content can be considered adequate because a litter of six young at 3 weeks contains from one-half to one-third, and even less, of the total calcium intake of the mother rat when she consumes this ration during the nursing period.

The antirachitic agent employed was irradiated dried yeast which contained 1 rat unit of vitamin D per mg. The preparation of this irradiated yeast has been described in another publication (5). It was substituted for a corresponding amount of non-irradiated yeast at a level equivalent to 0.5 per cent of the weight of the diet. It was fed only *during the period of lactation*. The total intake of vitamin D was equivalent to 500 rat units for each 100 gm. of diet.

A large number of females ranging in age from 104 to 111 days and in weight from 188 to 252 gm. were set aside for this series of experiments. Their preexperimental nutritional history was the

same as that of the animals in the two maintenance series. Ten females, representative of the group, were reserved for controls. Others were placed with males to be bred. They were fed regular stock diet up to the time of the first parturition except, as noted later, in the case of the controls. After parturition they were placed on the basal ration with and without vitamin D. Vaginal smears of all animals were taken daily to determine the date when parturition might be expected. The females were segregated in individual cages shortly before giving birth to young; clean pine shavings were provided for bedding. The litters were not reduced in size. Lactation was of 3 weeks duration. All females were weighed weekly, and later daily after the 2nd week of pregnancy. All litters were weighed at birth, at the end of the 1st and 2nd week, and daily thereafter. Feed was kept accessible to the females at all times up to the 14th day of lactation. Inasmuch as the young open their eyes at about this time and then begin to forage for themselves, all feed except distilled water and bedding was then removed from the cages. During the course of four periods in the days following the females were taken from their young and allowed to feed in separate cages. This practice restricted the young to a diet of mother's milk. Consumption records of the females on such a restricted feeding schedule showed that they consumed on an average from 20 to 30 gm. daily.

Certain females, apparently normal, which gave birth to litters but failed to nurse their young were placed with males to be rebred, being maintained on the basal diet without additional vitamin D until their second parturition. This was done to determine the effect of two pregnancies without lactation under such conditions.

From time to time, a number of females from the group reserved for controls were placed on the basal diet without irradiated yeast for a period of 3 weeks. This procedure was the same as that used for the animals subjected to reproduction.

Females were taken for analysis, respectively, 3 weeks after parturition, at the time of their second parturition, or, as in the case of controls, after having been on the basal diet for 3 weeks. Young were taken for analysis at the same time as their dams.

Individual blood samples were obtained from the females and calcium was determined in them in the manner already described. The femurs of the females were removed for ash analysis. The

young used for analysis were killed with ether, their intestinal tracts were removed, weighed, and discarded, and the remaining tissues dried. The dried residues of each separate litter were placed in a porcelain dish and ignited in a muffle furnace to a white ash with the aid of concentrated nitric acid. The ash was dis-

TABLE III

Effect of One Pregnancy and One Lactation on Females on Modified Stock Diet

	Rat No.	Initial weight	Final weight	No. of young raised	Weight of young minus intestinal tract	Ca in 100 cc. serum	Weight of femurs	Weight of femur ash	Ash in femurs	Relation to average ash of controls
		gm.	gm.		gm.	mg.	gm.	gm.	per cent	per cent
Litters of 5	52	245	270	3	98	9.6	0.905	0.570	63.0	97
and less	10	220	223	5	107	11.8	0.774	0.477	61.8	81
	23	200	191	5	97		0.607	0.364	59.9	62
	33	230	230	5	139	11.8	0.856	0.533	62.4	91
	51	230	223	5	137	11.4	0.771	0.477	61.8	81
	58	200	184	5	104	12.6	0.659	0.396	60.0	67
Average..........		221	220	5	114	11.4	0.762	0.469	61.4	80
Litters of 6	30	188	212	6	124		0.787	0.474	60.1	81
and more	19	215	223	6	145	11.0	0.799	0.483	60.4	82
	38	225	242	6	131		0.763	0.467	61.1	79
	42	222	201	6	135	10.4	0.678	0.412	60.7	70
	39	247	225	7	182	10.6	0.799	0.493	61.5	84
	34	210	186	9	155	11.8	0.610	0.371	60.8	63
	37	210	238	9	194	10.4	0.713	0.427	59.8	73
	22	247	196	11	167	11.6	0.722	0.424	58.7	72
Average.........		220	215	7	154	10.9	0.734	0.444	60.4	75
Grand average...		220	217	6	137	11.1	0.748	0.456	60.9	78

solved in dilute hydrochloric acid, filtered, and made up to volume. Duplicate aliquots of the acid solution were used to determine total calcium by McCrudden's (10) method.

The data obtained in the pregnancy and lactation series are summarized in Tables III to VII inclusive. To make possible a more direct comparison of results of comparable females as listed

in Tables III and IV, we have grouped the data in the order of the size of the litters raised.

Tables III, IV, and V reveal a pronounced reduction in femur ash with lactation. When the average total femur ash values of

TABLE IV

Effect of One Pregnancy and One Lactation on Female Rats on Modified Stock Diet with Additional Vitamin D

	Rat No.	Initial weight	Final weight	No. of young raised	Weight of young minus intestinal tract	Ca in 100 cc. serum	Weight of femurs	Weight of femur ash	Ash in femurs	Relation to average ash of controls
		gm.	gm.		gm.	mg.	gm.	gm.	per cent	per cent
Litters of 5 and less	14	192	212	3	82		0.686	0.484	60.2	82
	43	222	246	3	76	12.6	0.859	0.548	63.8	93
	5	225	200	4	108	10.4	0.807	0.513	63.5	87
	15	212	210	4	108	10.0	0.765	0.480	62.6	82
	44	215	221	4	95	11.6	0.731	0.457	62.5	78
	18	192	222	5	113	9.3	0.805	0.498	61.8	85
	20	198	234	5	146	10.7	0.719	0.439	61.0	75
	27	220	225	5	130	10.9	0.760	0.478	62.8	81
	35	235	247	5	134	11.6	0.773	0.481	62.2	82
	41	220	207	5	127	13.4	0.795	0.497	62.5	85
	55	247	240	5	100	12.2	0.923	0.555	60.2	94
Average..........		216	224	4	111	11.3	0.784	0.494	62.1	84
Litters of 6 and more	28	195	207	6	116	13.2	0.697	0.418	59.9	71
	4	237	238	7	173	10.4	0.803	0.484	60.2	82
	13	195	239	7	164	10.4	0.721	0.439	60.8	75
	40	218	209	8	167	10.4	0.711	0.435	61.1	74
	53	210	210	8	211	10.4	0.718	0.426	59.5	72
	36	217	239	9	206	10.1	0.753	0.456	60.5	78
Average		212	223	7	173	10.8	0.734	0.443	60.3	75
Grand average...		214	224	5.4	133	11.1	0.766	0.476	61.4	81

virgin females is taken as 100 the loss in weight amounts to 25 or 35 per cent and even more. Most noteworthy is the apparent ineffectiveness of vitamin D in preventing these reductions. However, a greater constancy is evident in the total femur ash of females receiving additional vitamin D than in those not receiving

such additions.   This narrower range in values may be taken to reflect a more general adequacy of the diet when supplemented with vitamin D, because in general, it may be said that the more limiting the diet becomes with respect to some one component, the more likely it is that individual animals will respond differently.

It is further evident that the size of litters raised and the size of the females are factors which determine the magnitude of withdrawal of ash from the skeleton.   In general, the smaller the female and the larger her litter the greater the reduction; and again those females which received additional amounts of vitamin D showed a slightly greater conservation of total ash.

TABLE V

Analyses of Femurs and Blood of Control Female Rats on Modified Stock Diet

Rat No.	Initial weight	Final weight	Ca in 100 cc. serum	Weight of femurs	Weight of femur ash	Ash in femurs	Relation to average ash of total group
	gm.	*gm.*	*mg.*	*gm.*	*gm.*	*per cent*	*per cent*
61	195	222	12.1	0.914	0.602	65.9	103
62	210	247	11.9	0.890	0.578	64.9	99
63	235	266	12.3	0.987	0.649	65.7	111
64	235	249	13.0	0.872	0.559	64.1	95
65	202	233	13.3	0.898	0.566	63.0	97
67	217	233	12.4	0.934	0.598	63.9	102
68	237	245	12.8	0.949	0.623	65.6	106
69	205	229	11.7	0.853	0.552	64.6	94
70	227	240	11.9	0.808	0.527	65.2	90
Average...	218	240	12.4	0.901	0.584	64.8	

The blood calcium values reveal no demonstrable effect from the feeding of additional vitamin D, as they all show average values of 11.1 to 11.4 mg. per 100 cc. of serum, but it undoubtedly is significant that the average figure for the non-lactating rats is 12.4 mg.   Apparently, there obtained during lactation not an excessive mobilization of calcium but rather an excessive demand with which the mobilization could not keep pace.

Table VI which shows the data on the effect of vitamin D supplementation on the weight of calcium in the young reveals no difference.   There undoubtedly were operative other limiting factors than availability of calcium or phosphorus.

Here it should be noted that we have considered only such young as grew to be 3 weeks old. All young which were born were not successfully raised and our mortality on this restricted diet was high. The fourteen females which received no additional vitamin D during lactation gave birth to 108 young of which number sixteen were consumed by the females and four were removed from

TABLE VI

Effect of Modified Stock Diet with and without Additional Vitamin D on Calcium Content of Nursing Young

	Without additional vitamin D					With additional vitamin D			
Rat No.	No. of young	Average weight of young minus intestinal tract	Average weight of Ca in young	Ca in young	Rat No.	No. of young	Average weight of young minus intestinal tract	Average weight of Ca in young	Ca in young
		gm.	gm.	per cent			gm.	gm.	per cent
52	3	33	0.219	0.67	14	3	27	0.318	1.16
10	5	21	0.159	0.74	43	3	25	0.223	0.88
23	5	20	0.199	1.01	5	4	27	0.206	0.95
33	5	28	0.254	0.91	15	4	27	0.233	0.86
51	5	27	0.259	0.94	44	4	24	0.203	0.85
58	5	21	0.158	0.75	18	5	23	0.261	1.15
19	6	24	0.223	0.92	20	5	29	0.268	0.91
38	6	22	0.226	1.03	27	5	26	0.219	0.84
42	6	23	0.189	0.84	35	5	27	0.233	0.87
30	6	21	0.215	1.04	41	5	25	0.208	0.82
39	7	26	0.217	0.83	55	5	20	0.151	0.75
34	9	17	0.155	0.90	28	6	19	0.207	1.07
37	9	22	0.196	0.90	4	7	25	0.233	0.94
22	11	15	0.139	0.91	13	7	23	0.228	0.97
					40	8	21	0.168	0.80
					53	8	24	0.192	0.72
					36	9	23	0.121	0.53
Average...	6.3	21.7	0.194	0.893		5.5	24.2	0.210	0.886

the cages as dead. The seventeen females receiving additional vitamin D during lactation gave birth to 124 young, of which number twenty-three were consumed by the females and seventeen were removed from the cages as dead. Vitamin D certainly was not the limiting factor in this respect.

An analysis of Table VII, which presents the data obtained from

the females which were subjected to two pregnancies, discloses no increase in femur ash under our conditions, such as might have been expected from Goss and Schmidt's (9) findings. The range in total femur ash weights, femur ash percentages, and blood

TABLE VII

Effect of Two Pregnancies Uncomplicated by Lactation on Female Rats on Modified Stock Diet

| Rat No. | Initial weight | Final weight | No. of young | | Weight | | Ca in 100 cc. serum | Weight of femurs | Weight of femur ash | Ash in femurs | Relation to average ash in controls |
			1st litter	2nd litter	1st litter	2nd litter					
	gm.	*gm.*			*gm.*	*gm.*	*mg.*	*gm.*	*gm.*	*per cent*	*per cent*
1*	192	230	5	6		32		0.875	0.571	65.2	97
9*	225	284	5	4	33	25	11.3	0.891	0.576	64.6	98
12*	220	258	5	7		41	11.8	0.948	0.605	63.8	103
31*	212	269	4	8		41	11.5	0.995	0.645	64.8	110
45*	205	242	3	7	17	40	13.4	0.901	0.573	63.5	98
50†	227	264	9	7	52	40	14.0	0.951	0.613	64.4	105
60†	237	289	8	10	47	55	12.0	0.986	0.637	64.5	109
26†	215	230	6	8	38	52	13.2	0.803	0.520	64.7	89
16†	217	254	8	8	47	45	12.4	0.929	0.598	64.4	102
21‡	227	279	10	10	56	54	14.2	0.932	0.576	61.8	98
57‡	202	236	9	7	46	33	11.1	0.869	0.552	63.3	94
17‡	210	264	8	9	44	58	11.5	0.989	0.640	64.7	109
25‡	190	238	8	9	42	46	13.6	0.897	0.582	64.8	99
Average...	214	257	7	7	42	43	12.5	0.920	0.591	64.2	101

* Returned to breeding cage at once.

† Returned to breeding cage after 1 to 5 days, with young on non-rachitogenic ration.

‡ Returned to breeding cage after 2 to 6 days, with young on non-rachitogenic ration with additional vitamin D.

calcium values, as well as their respective averages, parallel those of the unbred controls in a striking manner.

DISCUSSION

While it has been known for some time that ordinary rickets in the rat can be produced only by the feeding of a low calcium diet, or one in which the Ca and P ratio is abnormally high, observations

on the effect of rickets-producing rations on the adult over a long
period are not numerous. As a matter of fact, so far as we know,
such observations have been limited entirely to low Ca experi-
ments. Our present observations demonstrate conclusively, not
only that the skeleton of adult rats can be impoverished in ash
by the feeding of a ration containing an excessive amount of cal-
cium, but also that this can be ameliorated through the agency of
vitamin D, and in males as well as females. However, it is note-
worthy that the amelioration does not extend to the production
of normal bone as produced on a stock diet. It is possible that
the intake of vitamin D may not have been high enough, because
it was limited to the quantity produced by direct irradiation of
the ration, which restricted the intake to approximately 1 to 2 rat
units per day. But it is a fact that on this same ration, we have
observed a distinct tendency to the production of bone of lower
ash content with young growing rats even when they received an
abundance of vitamin D. It appears as though all the effects of
feeding a rachitogenic ration such as Ration 2965, which carries a
disproportionate amount of calcium, cannot be corrected by
supplementation with vitamin D.

With regard to the rôle that vitamin D may play in the human
adult, it may be opportune here to relate the findings of Kelly
and Henderson (11). They have shown in their work on adult
African natives that when a slight excess of calcium over mainte-
nance requirements obtains, the daily administration of 15 cc. of
cod liver oil brings the subject into positive balance with an in-
crease in the retention of calcium of nearly fourfold. Apparently
then, there are conditions in adult man on a simple maintenance
basis which can be corrected by vitamin D administration; but
it must be stated that an exact counterpart of the condition pro-
duced in rats, i.e. by the excessive intake of Ca, has not been
demonstrated with man.

With respect to our data relating to excessive losses of skeletal
ash induced by lactation, and the effect of vitamin D, we are
apparently dealing with a situation which has its counterpart in
heavily milking cows. Though the milk is enriched in vitamin D
by the feeding of additional amounts of it (5), calcium balances
are not improved (12). In the light of this ineffectiveness of

vitamin D, we are forced to come more and more to the belief that in those animals, at least, factors other than vitamin D dominate the calcium losses during lactation.

We have already stated that contrary to the observations of Goss and Schmidt, we did not demonstrate an actual storage of calcium in the maternal tissues during pregnancy. But it should be stated that the experimental conditions were not exactly the same in the two experiments. In the first place, we subjected our females to two pregnancies in succession, in an attempt to accentuate the effect, and in the second place, we used only those females which failed to nourish their first litters; others were continued for lactation studies. It is possible, therefore, that our females were not strictly normal in their calcium relations or that the second pregnancy was too severe a strain. However, it must also be considered possible (a) that the storage observed by Goss and Schmidt took place more extensively in other tissues than the femurs, to which our analyses were limited, and (b) that the animals used by Goss and Schmidt were not adequately supplied with calcium reserves when put on experiment.

It is obvious that when an attempt is made to compare experiences from different laboratories, sight must not be lost of pre-experimental factors, specifically those relating to the nutritive state of the animal. It, of course, is well known that pregnancy constitutes a tremendous stimulus to the maternal organism, but studies of its specific effects are not sufficiently numerous to allow generalization.

SUMMARY

Adult male and female rats kept on a rachitogenic diet which contained an excessive amount of calcium in relation to phosphorus, lost mineral elements from their skeletons. This loss was reduced but not prevented by the addition of vitamin D. Two successive pregnancies uncomplicated by lactation did not decrease the ash content of femurs. Femur ash content was markedly reduced by lactation even when generous amounts of vitamin D were incorporated in a diet which carried liberal amounts of calcium and phosphorus and some vitamin D. The calcium content of new born young was fairly constant irrespective of vitamin D additions to the mother's diet.

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STUDIES ON GLUTELINS

VII. CYSTINE, TRYPTOPHANE, AND TYROSINE CONTENT OF GLUTELINS

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The chemical composition of glutelins reported in our previous publications[1] in this series was determined by means of the Van Slyke method. As to the individual amino acid content, this method gives information only in regard to arginine, histidine, lysine, and cystine. Data on the basic amino acid content of proteins obtained by the Van Slyke method by Osborne, etc. (1) and recently by Vickery (2) and by Calvery (3), agree fairly well with the figures obtained gravimetrically by direct separation. Cystine, however, determined colorimetrically in various proteins by Sullivan and Hess showed differences from those determined by Van Slyke's method (4). The importance of knowing the correct percentages of the amino acids in proteins, such as glutelins, which are present in practically all cereals, is emphasized by the fact that four of them, histidine, lysine, cystine, and tryptophane, are nutritionally essential for growth and maintenance. Tyrosine also is considered by some as one of the essential amino acids, although evidence is far from being conclusive.

Of the three amino acids considered, cystine is probably the only one affected by the alkali used in the preparation of glutelins. The other two, tryptophane and tyrosine, are known to be stable in alkali. The use of alkali is unavoidable since we have no other means of extracting glutelins from cereals. Whatever loss of cystine may occur during the preparation remains undetermined and cannot be estimated. The loss that results from heating free cystine in alkaline solution cannot be applied to estimate the

[1] Specific publications are referred to in Table II.

loss occurring when cystine is a part of the protein molecule. Even losses of cystine produced by alkali in proteins other than glutelins would be merely an approximation. A higher percentage of cystine obtained by a colorimetric method other than Sullivan's is not necessarily more nearly correct unless it is proved conclusively that the color reaction used for cystine is specific. The cystine lost by contact with alkali during the preparation of a glutelin cannot be recovered by the same reagent which is specific for cystine itself.

<div align="center">EXPERIMENTAL</div>

The methods used for the preparation of glutelins are described in our previous publications[1] in this series. The colorimetric methods used were those of Sullivan (4) for cystine, May and Rose (5) for tryptophane, and Folin and Ciocalteu (6) for tyrosine. Some changes in the tryptophane and cystine methods were introduced, which will be discussed in detail.

Tryptophane Determination—May and Rose have arbitrarily chosen for the color development 24 hours at 35° and 48 hours at room temperature. Apparently they assumed that the intensity of color develops in standard (casein) and unknown (protein under investigation) at a similar rate and reaches the maximum at the time interval they adopted. Holm and Greenbank (7) have shown that the time allowed by May and Rose for the color development is too short. They thought this fault could be avoided by making readings from time to time ". . . until the colors of the unknowns reached maximum intensity." In 1925 we studied the rate of color development of tryptophane in several different proteins with the object of establishing a more reliable technique, keeping in mind that the maximum color of the standard solution should be compared with the maximum color of the unknown. Boyd (8), in 1929, published experiments of a similar nature. Although we finished our problem and applied our results on several occasions, we have postponed publication until now.

The rate of color development, in the dark, at 37°, which is the temperature we adopted, differs from that of Holm and Greenbank. It was found that when using tryptophane, as well as casein and the other proteins, the maximum color was reached on the 5th day (Table I), just half the time specified by Holm and

Greenbank. The rate of color development is irregular up to the 5th day, when it reaches the maximum. Because the fading also varies in different proteins, the maximum reading shown by the unknown may not represent the correct one.

To compare the daily color change a colorimeter with Lovibond glass standard (combination of blue 4.8 and red 0.88) was used, which matched the color developed with 2 mg. of tryptophane in 100 cc. of hydrochloric acid (1:1) and 1 cc. of Ehrlich's reagent, according to the method of May and Rose. The same colored glass standard was used for casein. The maximum reading in mm.

TABLE I

Rate of Color Development in May-Rose Tryptophane Method Expressed in Mm. As Measured by Lovibond Color Standards (Blue 4.8 and Red 0.88)

Date	Material under investigation (moisture- and ash-free)	Color development in					
		2 days	3 days	4 days	5 days	6 days	7 days
		mm.	mm.	mm.	mm.	mm.	mm.
Oct. 5, 1926	2 mg. tryptophane	18.7	12.7	11.3	10.9	12.0	12.5
June 10, 1927	2 " "		11.4	11.4	10.8	11.6	
Sept. 30, 1926	2 " "	13.7	11.9	11.1	10.7		
Feb. 12, 1927	0.1 gm. casein	11.4	10.9	10.9	10.2	10.9	12.1
" 21, 1927	0.1 " "	11.6	10.5	10.4	10.4		
" 12, 1927	0.8763 gm. α-glutelin of wheat	13.5	12.5	12.6	11.3	12.3	13.4
" 12, 1927	0.9117 gm. rice glutelin	18.5	15.7	13.7	12.9	13.7	14.1

was observed on the 5th day. Determinations made on a solution containing 2 mg. of tryptophane gave an average reading on the 5th day of 10.8 mm. Similar determinations made on 0.1 gm. of moisture- and ash-free casein gave an average reading of 10.3 mm. These data show that casein contains 2.09 per cent tryptophane. In all our work this figure was used as a basis for calculating tryptophane percentages since we used casein as a standard as recommended by May and Rose.

Cystine Determination—The preparation of the protein hydrolysates for the color test according to Sullivan's method is admittedly tedious and may easily be simplified by introducing certain modi-

fications which are used in the Folin and Marenzi (9) method.
0.5 to 1.0 gm. of air-dried material was weighed in a test-tube and
the material transferred into a dry 300 cc. round bottom flask.
20 cc. of a 6 N aqueous solution of sulfuric acid and a few glass
beads were added, and the mixture was boiled gently over a gas
flame for 18 or 20 hours.   A Hopkins condenser that fitted closely
into the neck of the flask was used.   It is advisable to shake the flask
gently during the 1st hour of heating.   At the end of the boiling
period the condenser is rinsed with distilled water, the contents of
the flask are cooled, and approximately 95 per cent of the acidity
is neutralized by adding with shaking and cooling, drop by drop,
an approximate 40 per cent solution of sodium hydroxide.   (The
quantity of alkali required may be estimated by measuring 20 cc.
of the 6 N sulfuric acid and titrating it with the alkali, sodium ali-
zarin sulfonate being used as an indicator.)

The hydrolysate is now transferred quantitatively into a 100 cc.
volumetric flask, and the flask is filled to the mark with distilled
water.   The hydrolysate is decolorized by pouring it into a beaker
containing 2 gm. of kaolin and shaking gently for 3 minutes, after
which it is filtered.   The filtrate is still very slightly colored, which,
however, does not interfere with the accuracy of the test.

5 cc. of the filtrate are removed to determine the amount of
alkali required to reach approximately pH 3.5, thymol blue being
used as an indicator (until the yellow color is reached).   Another
5 cc. is measured into a 25 cc. volumetric flask, and the required
alkali is added drop by drop.   Sullivan's instructions are now fol-
lowed.   Add 2 cc. of 5 per cent aqueous sodium cyanide, mix, and
after 10 minutes, add 1 cc. of a 0.5 per cent aqueous solution of 1.2
naphthoquinone-4-sodium sulfonate, shake it for 10 seconds, and
add at once 5 cc. of a 10 per cent solution of anhydrous sodium
sulfite in 0.5 N sodium hydroxide.   After 25 minutes 2 cc. of a
5 N sodium hydroxide and 1 cc. of a 2 per cent solution of sodium
hydrosulfite ($Na_2S_2O_4$) in 0.5 N sodium hydroxide are added, the
flask is filled to the mark with distilled water, and the solution is
compared with a cystine standard prepared similarly and simulta-
neously with that of the unknown.   The strength of the standard
solution selected should be near that of the unknown.

All the reagents are prepared in quantities required for immediate
use.   Cystine in the kaolin-clarified hydrolysates is stable and may

safely be kept in a refrigerator for 2 or 3 days, but this is not advisable for the material obtained according to Sullivan's method.

Since the method of preparation of our glutelin samples differs from that used in the past, comparison of data on their amino acids with data obtained by other methods is unjustified. The figures in Table II represent averages of at least two determinations. The chief protein of rice is glutelin, and in wheat, glutelin is generally second to gliadin. It is interesting to note, therefore, the outstanding quality of wheat and rice among the cereals, judging from the high percentages of the indispensable amino acids in their glutelins. In corn and rye, although the amount of glutelin is rather small, it supplies nutritionally some tryptophane, which is

TABLE II

Amino Acids in Moisture- and Ash-Free Glutelins

	Cystine	Tryptophane	Tyrosine
	per cent	*per cent*	*per cent*
α-Glutelin of hard wheat (10)......	1.160	2.24	5.69
" " soft " (10).....	1.354	2.12	5.07
" " wheat (from Ceresota commercial flour) (10)..........	1.440	2.04	5.30
Glutelin of rye (11)...............	0.758	1.80	4.49
α-Glutelin of barley (11)..........	0.904	0.4–0.5	3.70
Glutelin of rice (12)..............	1.170	1.76	5.79
α-Glutelin of corn (13)............	0.516	2.07	4.99
Glutelin of oats (14)..............	0.805	1.85	4.43

missing entirely in zein and is present only in a small percentage in the rye prolamine.

SUMMARY

The cystine, tryptophane, and tyrosine content of glutelins in the cereals of wheat, rice, corn, rye, barley, and oats, are given. Some modifications are described for the colorimetric determination of cystine by Sullivan's method and for tryptophane by May and Rose's method.

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THE RATE OF AUTOXIDATION OF OXIDATION-REDUCTION SYSTEMS AND ITS RELATION TO THEIR FREE ENERGY*

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In a previous paper (1) it was shown that there was a relation between the catalytic power of reversible dyes upon cellular oxygen consumption, and the E'_0 of the dye. Since in those experiments the oxidative reactions took place in such a heterogeneous system as the cell, it was decided to study the velocity of oxidation by atmospheric oxygen of the dyes used in the experiments mentioned above, extraneous influences (such as catalysts, change in ionic strength, etc.) being avoided as far as possible, and to see if, in such homogeneous solutions, the same relation exists between the potential of the dyes and the speed of oxidation. From the experiments presented in this paper it can be seen that the speed of oxidation of a leuco dye by molecular oxygen seems to depend on the oxidation-reduction potential of the dye, provided catalysts are not present. Even when the presence of catalysts accelerates the reaction beyond the expected values, there has been found the same relationship between the free available energy of the particular system and the rate of its oxidation.

Although it is justly assumed in thermodynamics that there is, in general, no necessary relationship between the speed and free energy of a reaction, the conclusions drawn from the experiments to be related in this paper are in accord with some evidence already in the literature. Conant (2), in studying the "apparent reduction potential" of reducing substances capable of an irreversible reduction (a number of azo dyes, nitro compounds, and unsaturated

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1,4-diketones) by adding an equimolecular mixture of the reduced and oxidized forms of a reversible reductant, found that the reduction in a homogeneous solution of such compounds is a function of the potential of the reducing agent. La Mer and Temple (3), studying the rate of oxidation of hydroquinone under the catalytic influence of manganous salts, have found that it is a simple linear function of the available free energy of the system. Voegtlin, Johnson, and Dyer (4) observed that under standard conditions, the reduction time of the various oxidation-reduction indicators decreases with an increase in the electrode potential of the indicator, and concluded that "the reduction time is approximately a logarithmic function of the electrode potential." More recently, Michaelis and Smythe (5) have reported that for a series of iron compounds the autoxidizability of the ferro compound at a given pH "closely parallels the normal oxidation-reduction potential of the system ferro compound-ferri compound at the same pH."

Electrometric Method for Determining Rate of Oxidation of a Reversible System

It was first decided to determine the rate of oxidation of the reduced dye by manometric methods. The dye, which was kept in a stopperd flask provided with a gas inlet and outlet, was reduced by means of platinized asbestos and hydrogen. A stream of nitrogen was passed through afterwards. A burette with the stop-cock at the upper end was used as gas outlet and was immersed in the solution to receive the dye, which had previously been filtered through a Jena glass filter. In Fig. 1 is given the rate of oxidation of certain reduced dyes, as obtained by this method. Cresyl blue which in this case has the more positive E'_0 value is oxidized at very low speed as compared to the oxidation of indigotetrasulfonate. Phenolindophenol with an E'_0 more positive than the latter and more negative than cresyl blue is oxidized at a speed higher than that of cresyl blue and lower than that of indigotetrasulfonate. But, although it is possible to measure the speed of oxidation of leuco dyes which are slowly oxidized, this method offers difficulties when studying leuco dyes which oxidize more easily. For these reasons, it was decided in subsequent experiments to measure the speed of oxidation by means of the potentiometer. La Mer and Temple (3) were the first to apply an

electrometric method for following the autoxidation of hydroquin-
one. The method is based on the measurement at definite time
intervals of the E.M.F. of a cell containing the reversible system in
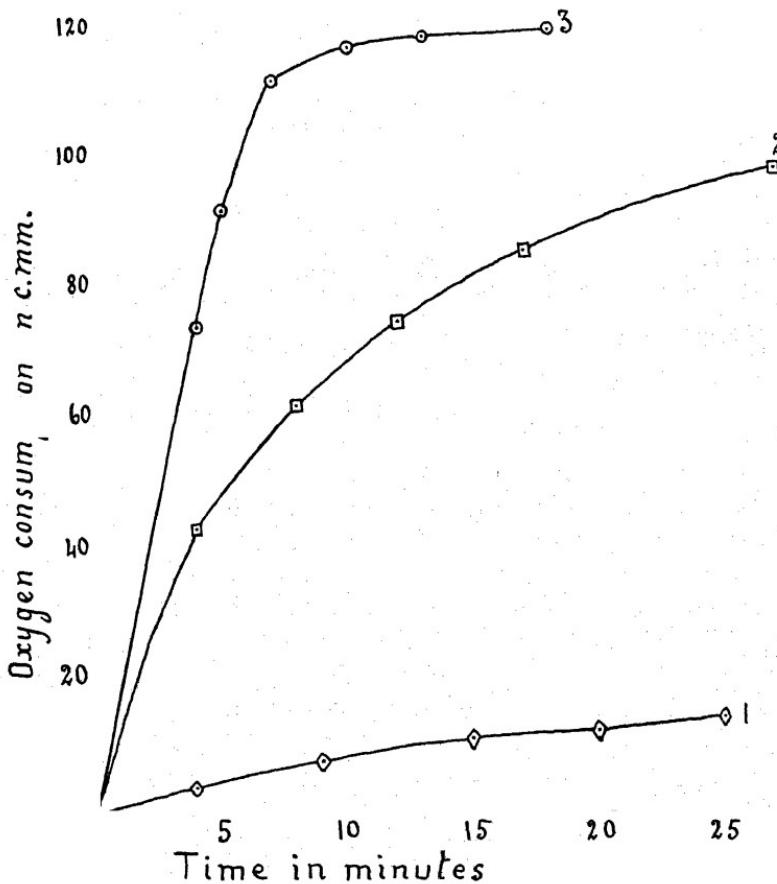
FIG. 1. The rate of oxidation of reversible dyes as measured by the con-
sumption of oxygen; Curve 1 cresyl blue at pH 4.18 ($E'_0 = +0.212$ volt),
Curve 2 phenolindophenol at pH 7.78 ($E'_0 = +0.177$ volt), Curve 3 indigo-
tetrasulfonate at pH 4.18 ($+0.115$ volt).

its reduced form, and the calculation of concentrations of these
substances by means of the known thermodynamic relationships.
The method is, as shown by Temple (6) more valid, more flexible,
and of a much higher precision than the gasometric method.

If an "inert" electrode (gold, bright platinum, carbon, tungsten, mercury) be placed in a solution of constant (H^+), containing a reversible oxidation-reduction system, there will almost immediately be assumed a stable potential which is determined by the ratio of oxidant and reductant. The relation is given by the well known Peters' equation (7)

$$E_h = E'_0 - \frac{RT}{nF} \ln \frac{(S_o)}{(S_r)}$$

Where E_h is the E.M.F. of the system referred to the normal hydrogen electrode, E'_0 the E.M.F. when the ratio of oxidant to reductant is equal to one, R the gas constant, T the absolute temperature, n the number of electrons, F the faraday, (S_o) concentration of oxidant, and (S_r) concentration of reductant.

If α is the degree of oxidation

$$E_h = E'_0 - \frac{RT}{nF} \ln \frac{\alpha}{1 - \alpha}$$

The following technique has been employed in our experiments. The dye in its oxidized form was dissolved in a small amount of water (8 cc.) and buffered with Sörensen's citrate buffers (90 cc.). 2 cc. of colloidal palladium (1 per cent aqueous solution of Kalle's colloidal palladium) were added and the whole introduced into the reaction vessel (Fig. 2). Two electrodes, both of pure gold, or one of gold and the second of graphite, were always used, although most of the measurements were followed with only one electrode. The other half-cell was a saturated calomel electrode and the connections made through an agar KCl bridge. The experiments were performed at 25°.[1] The dye was reduced by passing a stream of purified hydrogen through stop-cock A, keeping stop-cock B (which connects the reaction vessel to the air) closed. As soon as the dye was completely reduced, the hydrogen stream was replaced by purified nitrogen which was kept bubbling for about 1 hour. Then, stop-cock A was closed and stop-cock B was opened. At this moment, stirring was begun and oxygen at

[1] The values for the E'_0 of the different dyes used in these experiments have been determined at 30°. As there have not yet been made studies on the temperature coefficients, the E'_0 at 30° has been taken as valid at 25°.

atmospheric tension was bubbled into the reaction vessel at a rate carefully controlled by a flowmeter, where a column of Brodie's solution (23 gm. of NaCl, 5 gm. of sodium taurocholate, 500 cc. of water, plus some drops of a saturated alcoholic solution of thymol) indicated the pressure of the air passing through. A reasonable space of time elapsed before the E.M.F. reached a ratio corresponding to 2 per cent of oxidation, which was chosen as zero time. From
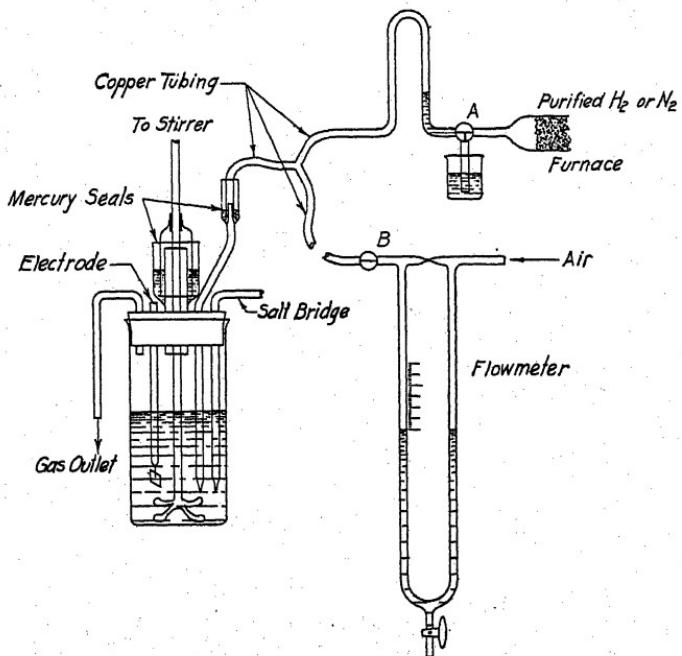
FIG. 2. Schematic drawing of the apparatus used for measuring electrometrically the rate of oxidation by atmospheric oxygen of reduced dyes. No attempt has been made to draw it to scale.

this moment, the E.M.F. was measured at close intervals until a ratio of 50 per cent oxidation, i.e. the E'_0 of the system, was reached. The experiment was terminated at this point.

Although the potentials obtained in solutions containing reversible systems may not agree closely when the system is poorly poised, in these experiments, where the readings were started only when there was 2 per cent oxidation, the potentials obtained with electrodes of different nature, such as gold and carbon, were always

, in close agreement, showing that the influence of molecular oxygen on these potentials was negligible. In Fig. 3 are plotted data of the rate of oxidation of toluylene blue (at pH 5.865), where pure gold and graphite were used as electrodes and the readings made from both of them. As soon as 50 per cent oxidation was reached, air flow was stopped and nitrogen flow was started. The potential became 9 millivolts more positive, as would be expected, since there still was oxygen in the reaction vessel, and then it remained constant, giving in both electrodes identical readings. The graph-
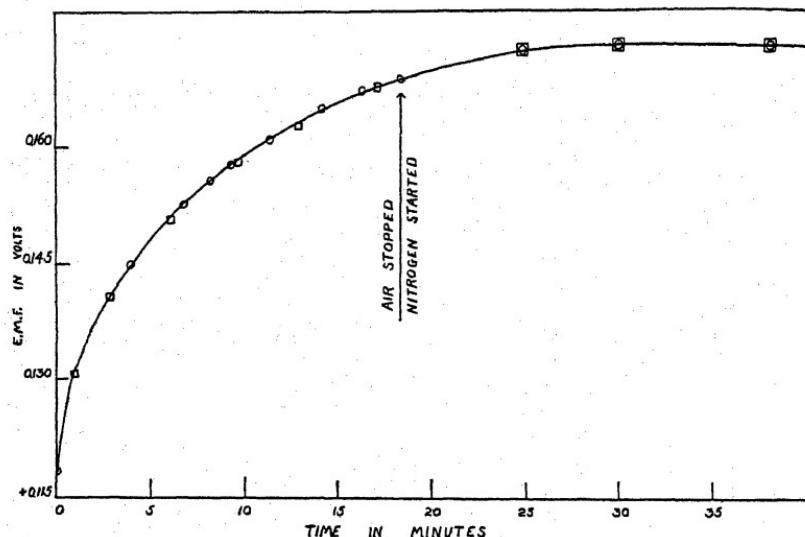
FIG. 3. Velocity of autoxidation of reduced toluylene blue, measured electrometrically; ⊙ with gold electrode, ☐ with graphite electrode.

ite electrode has the disadvantage of being rather sluggish when the system is poorly poised, as has been shown in a previous paper (8). In all the experiments here reported the rate of air flow was kept at a speed of 2 cc. per minute.

The concentration of colloidal palladium has no influence on the rate of oxidation. The amount of the catalyst (0.1 per cent of colloidal palladium) has been changed from 10 to 2 cc. without any change in results.

It is well known that the rate of oxidation of reversible systems can be greatly accelerated by the addition of catalysts. The very

slow rate of autoxidation of hydroquinone by atmospheric oxygen in neutral and acid solutions, is greatly accelerated under the influence of manganous ions (Bertrand (9)), salts of iron, copper, vanadium, and uranium (Aloy and Valdiguié (10)). The rate of autoxidation of methylene blue in acid solutions, is also greatly increased by the addition of either copper (Reid (11)) or iron (Macrae (12)). In order to avoid the effect of catalysts, only purified dyes were used. As some anions have also, at times, an accelerating or retarding influence, the nature of the buffer was kept constant (Sörensen's citrate buffer).

TABLE I

Oxidation of Reduced Toluylene Blue by Atmospheric Oxygen

Dye concentration 0.001 M; 0.1 M citrate buffer at pH 4.663; 90 cc. of buffer + 7 cc. of H_2O + 3 cc. of 0.1 per cent colloidal Pd; $T = 25°$.

Au-Pt electrode, Experiments I and II	Time		Oxidation
	Experiment I	Experiment II	
	min.	*min.*	*per cent*
+0.191	0	0	2
+0.204	2.26	2.19	5
+0.213	5.19	5.10	10
+0.219	8.09	8.17	15
+0.224	11.26	11.30	20
+0.228	14.50	14.58	25
+0.231	18.00	18.04	30
+0.234	21.41	21.53	35
+0.237	26.07	26.14	40
+0.239	29.33	29.38	45
+0.242	35.26	35.34	50

The concentration of the dye was 0.001 M.

The fundamental researches of Clark and his coworkers (13), who prepared and determined the potentials of a series of reversible oxidation-reduction dyes with unexcelled technique and the most precise manner, have made possible this study of the autoxidation of reversible dyes and its relation to their potentials. In this paper is reported the comparative rate of autoxidation of the following dyes: phenolindophenol, o-cresolindo-2,6-dichlorophenol; 1-naphthol-2-sulfonate-indophenol; toluylene blue; cresyl blue; 1-naphthol-2-sulfonate-indo-2,6-dichlorophenol; and indigo-

tetrasulfonate. All these dyes were generously provided by Professor W. Mansfield Clark, and some of them purified according to the methods given by him and his coworkers.

In order to see that neither reduction by colloidal palladium nor oxidation by atmospheric oxygen had produced irreversible changes in the dye system, the leuco dye oxidized by air was once more reduced and the rate of oxidation measured. Table I shows that reproducible results are obtained.

The oxidation of these dyes has been followed at constant pH, namely pH 5.86, with the exception of o-cresolindo-2,6-dichlorophenol, where the oxidation was performed at pH 6.65.

TABLE II

Rate of Autoxidation of Reversible Oxidation-Reduction Dyes. Oxidant, Atmospheric Oxygen, at a Rate of 2 Cc. per Minute

Dye concentration 0.001 M in citrate buffers; $T = 25°$.

Dye (98 per cent reduced)	E'_0	pH	Time required to reach 50 per cent oxidation
	volt		*min.*
Phenolindophenol..................	+0.297	5.865	59.40
o-Cresolindo-2,6-dichlorophenol ...	+0.207	6.65	26.08
1-Naphthol-2-sulfonate-indophenol.	+0.191	5.864	22.37
Toluylene blue....................	+0.169	5.865	17.22
Cresyl "	+0.096	5.865	7.55
Indigotetrasulfonate..............	+0.009	5.783	3.58

In Table II is given the time required to oxidize the reduced dyes (98 per cent reduced) to 50 per cent oxidation (the E'_0 of the dye). In Fig. 4 is plotted the E'_0 of the dyes against the logarithm of the time required to reach 50 per cent oxidation. It can be clearly seen that there exists a relation between the E'_0 of the dye and the speed of oxidation of the leuco dye. This relation is a logarithmic function of the time required for oxidation. The same relationship can be observed when the E'_0 is changed by a change of the pH of the buffer. Such a study has been carried out with toluylene blue, the pH having been changed from 2.90 to 5.86.[2] As can be seen in Fig. 5, the same relation has been found

[2] The E'_0 of toluylene blue shows between these pH limits a "0.06 slope," as seen from Philips, Clark, and Cohen's data (14). It is reported in this

between the E'_0 of the dye and the logarithm of the time required to oxidize the leuco dye from 2 per cent to 50 per cent oxidation. Systems having extremely high positive potentials have been reported by Clark and coworkers. o-Tolidine has an E'_0 of $+0.634$ at pH 4.88 and benzidine, an E'_0 of $+0.672$ at the same pH. Their oxidation ought to be performed at very low speed. In fact the rate of oxidation of these reversible systems was too low to be measured.

Exceptions to this relation between the potential of a reversible system and the speed of oxidation have, indeed, been found.
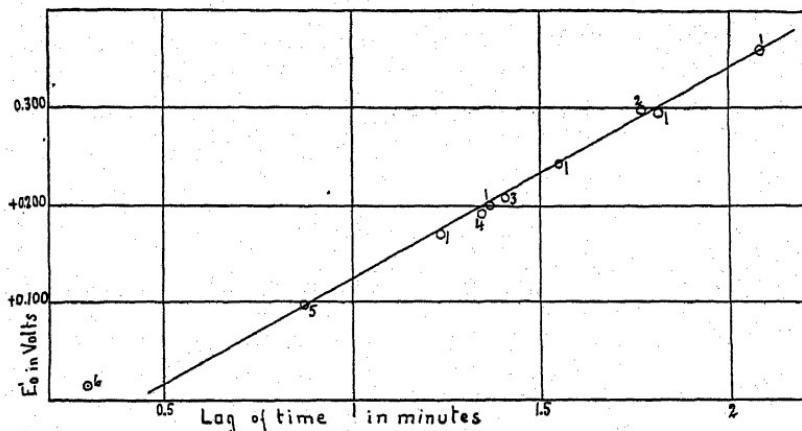
Fig. 4. Velocity of autoxidation of reversible oxidation-reduction systems. The reduced dyes have been oxidized by atmospheric oxygen from 2 per cent to 50 per cent oxidation (E'_0 of the dye). 1 indicates toluylene blue; 2, phenolindophenol; 3, o-cresolindo-2,6-dichlorophenol; 4, 1-naphthol-2-sulfonate-indophenol; 5, cresyl blue; 6, indigotetrasulfonate.

Among the dyes, 1-naphthol-2-sulfonate-indo-2,6-dichlorophenol is oxidized at higher speed than predicted. At pH 5.86 ($E'_0 +$ 0.205) it should have been oxidized in 23 minutes; the oxidation to 50 per cent was performed in 13 minutes, 50 seconds. It was found that this preparation was only 72 per cent pure dye. The

paper that toluylene blue is easily transformed into neutral red which has a very negative potential. The presence of neutral red therefore should not interfere with the measurements of the toluylene blue system.

possibility of the presence of a catalyst may be suggested as an explanation for its anomalous behavior. Hydroquinone is another exception. At pH 5.86 ($E'_0 + 0.347$) the time of oxidation would be predicted to be 111 minutes. The rate of oxidation was in fact very slow.
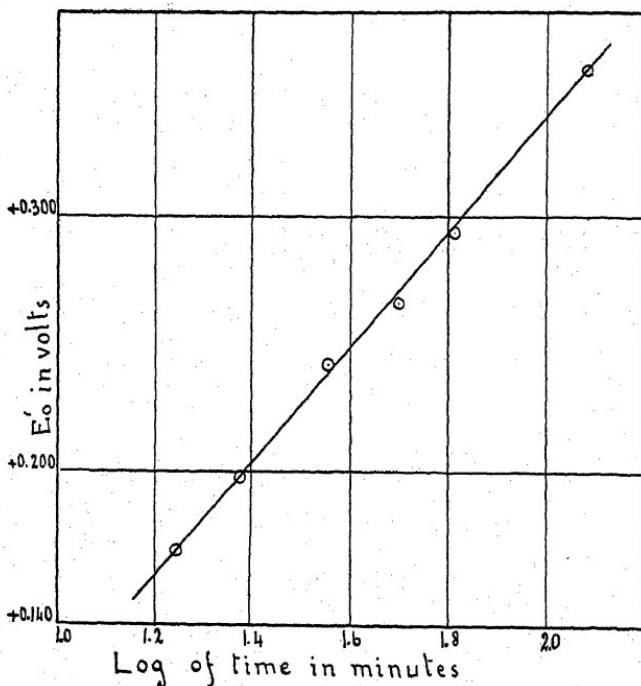
FIG. 5. The rate of autoxidation of toluylene blue at different pH. Ordinate, E'_0 of the dye; abscissa, logarithm of time (in minutes) required to oxidize the leuco dye from 2 per cent to 50 per cent oxidation.

Rate of Oxidation of Reversible Dyes

If there is a relation between the available free energy of an oxidation-reduction system and the speed of oxidation, this relation must be evident when one studies the rate of oxidation of individual systems. In such a case the disturbing factors mentioned above (catalysis, the effect of H^+, etc.) will exert identical influence. In all the cases where the speed of the oxidation has allowed multiple measurements, this relationship between the available free energy of the system and the speed of oxidation has been found.

The same logarithmic function exists between the potential (expression of the free available energy of the system) and the rate of oxida-
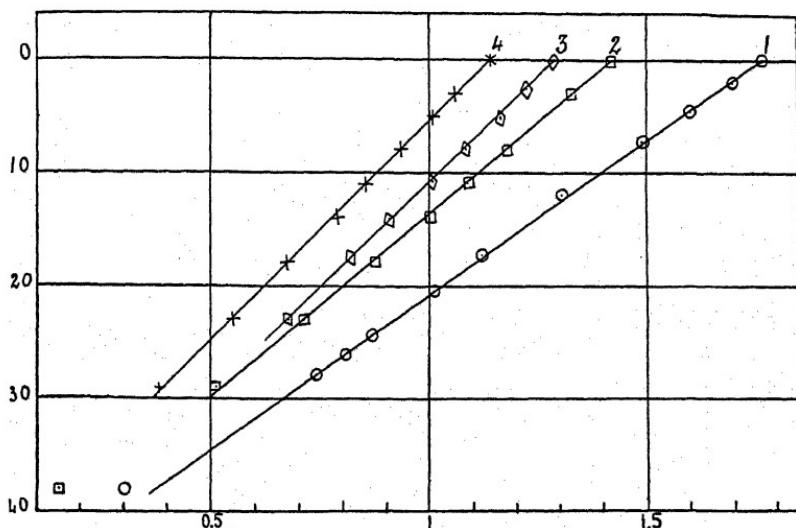
FIG. 6. The rate of autoxidation of reversible dyes. Abscissa, logarithm of time (in minutes) required to oxidize the reduced dye; ordinate, $E'_0 - \frac{RT}{2F} \ln \frac{\alpha}{1 - \alpha}$ expressed in millivolts. Curve 1 phenolindophenol, Curve 2 o-cresolindo-2,6-dichlorophenol, Curve 3 methylene blue, Curve 4 1-naphthol-2-sulfonate-indophenol.
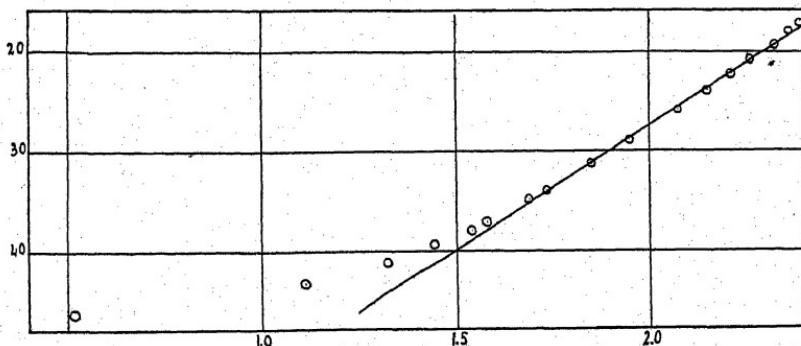
FIG. 7. The velocity of autoxidation of hydroquinone at pH 7.57. Ordinate, $E'_0 - \frac{RT}{2F} \ln \frac{\alpha}{1 - \alpha}$ expressed in millivolts; abscissa, logarithm of time (in minutes) required to oxidize hydroquinone from 2 per cent.

tion as can be seen in Fig. 6.   In certain cases, as in the oxidation of toluylene blue, o-cresolindo-2,6-dichlorophenol, 1-naphthol-2-sulfo-nate-indo-2,6-dichlorophenol, the rate of oxidation can be expressed by the equation for reactions of the first order (Table III).   Neill and Hastings (15) and quite recently Brooks (16) have found that the

TABLE III

Rate of Oxidation by Atmospheric Oxygen

Reduced o-cresolindo-2,6-dichlorophenol Dye concentration 0.001 M; buffer, 0.1 M citrate-NaOH, pH 6.65; $T = 25°$			Reduced toluylene blue Dye concentration 0.001 M; buffer, 0.1 M citrate-HCl, pH 4.663; $T = 25°$		
Oxidation	Time	$k = \frac{1}{t}\log\frac{a}{a-x}$	Oxidation	Time	$k = \frac{1}{t}\log\frac{a}{a-x}$
per cent	*min.*		*per cent*	*min.*	
2	0		2	0	
10	3.25	1.14×10^{-2}	10	5.32	8.79×10^{-3}
15	5.20	1.19×10^{-2}	15	8.15	8.84×10^{-3}
20	7.53	1.17×10^{-2}	20	11.43	8.67×10^{-3}
25	10.03	1.16×10^{-2}	25	14.83	8.62×10^{-3}
30	12.50	1.17×10^{-2}	30	18.00	8.82×10^{-3}
35	15.37	1.16×10^{-2}	35	21.68	8.85×10^{-3}
40	18.55	1.15×10^{-2}	40	26.12	8.71×10^{-3}
45	21.50	1.17×10^{-2}	45	29.55	8.99×10^{-3}
50	26.08	1.13×10^{-2}	50	35.43	8.73×10^{-3}
Reduced 1-naphthol-2-sulfonate-indo-2,6-dichlorophenol Dye concentration 0.001 M; buffer, 0.1 M citrate, pH 5.86; $T = 25°$			Reduced methylene blue Dye concentration, 0.0001 M; buffer, 0.1 M citrate-HCl, pH 2.68; $T = 25°$		
2	0		2	0	
10	2.40	1.95×10^{-2}	15	4.80	1.504×10^{-2}
15	3.58	2.02×10^{-2}	20	6.62	1.496×10^{-2}
20	4.75	2.30×10^{-2}	25	8.22	1.547×10^{-2}
25	6.25	2.04×10^{-2}	30	10.28	1.542×10^{-2}
30	7.18	2.21×10^{-2}	35	12.22	1.570×10^{-2}
35	8.66	2.21×10^{-2}	40	14.60	1.560×10^{-2}
40	10.28	2.21×10^{-2}	45	16.90	1.576×10^{-2}
45	11.63	2.29×10^{-2}	50	19.38	1.598×10^{-2}
50	13.75	2.25×10^{-2}			

rate of oxidation by molecular oxygen of the system hemoglobin ⇌ methemoglobin is also monomolecular.   The oxidation of leuco-methylene blue could not be studied at the concentrations used for the other dyes, in view of the extremely low solubility of the leuco dye (its solubility at pH 2.9 has been estimated by Clark,

Cohen, and Gibbs to be 0.0005 to 0.0006 M). Therefore, the dye was used at 0.0001 M concentration. It was dissolved in citrate buffer at pH 2.68. Here also the same relation exists between the free available energy of the system and the rate of oxidation as can be seen in Fig. 5. The rate of oxidation can be expressed by the equation of the so called monomolecular reactions (Table III).

The rate of oxidation of hydroquinone was studied by La Mer and Temple (3), with manganous salts as catalyst for the reaction. In these experiments, hydroquinone was oxidized in absence of catalysts at pH 7.57 (Sörensen's phosphates). The hydroquinone (0.001 M) was kept in a spoon while the solution was deaerated. The slow rate of oxidation allowed careful potentiometric measurements. As can be seen in Fig. 7, here, too, the rate of oxidation is a function of the logarithm of time. Because of the sluggishness of the rate of oxidation, it was followed only until 20 per cent oxidation was reached.

DISCUSSION

"At one time the equation Reaction velocity $\cdot \dfrac{\text{Chemical affinity}}{\text{Chemical resistance}}$ was used in a qualitative and descriptive way, but it is really rather misleading, because there is no correlation whatever between chemical affinity, as measured by the maximum work, and speed of reaction . . . . Affinity depends solely upon initial and final states. Velocity of reaction, on the other hand, depends upon the nature of some specific reactive state in which the molecules become capable of rearrangement" (Hinshelwood (17)).

Thus can be summarized the position taken by students of chemical kinetics whenever an attempt is made to correlate free energy to speed of reaction. This position has sound basis in well known thermodynamic assumptions. In the case of either hydroquinone or methylene blue, the speed of oxidation by oxygen will be increased by the addition of a catalyst, although the free energy of the system independent of the intermediate steps will depend only on the ratio $\dfrac{\text{(reductant)}}{\text{(oxidant)}}$. A comparison of the velocity of oxidation in different systems under such circumstances is, of course, untenable. But, when the comparison is restricted to simple reversible systems which proceed along the most direct path and are strictly reversible at every stage, then it would perhaps be possible to find a relation between free energy and speed of reaction.

In the systems under consideration the reaction, reductant$^-$ $\rightleftarrows$ oxidant$^+$ + $2e$ is strictly reversible. The function of the oxygen molecule is simply to act as an acceptor for the electrons liberated in the oxidation ($2e$ + $O_2 \rightarrow O_2^=$). The fundamental process, transfer of electrons, is of the most elementary character and to admit a relation between the free energy and affinity seems in such cases logical. The experiments which have been described in the present paper seem to give some basis to such an assumption. Several dyes have been taken, and the speed of oxidation has been measured electrometrically from the 2 per cent oxidation to 50 per cent oxidation. It has been found that in these particular cases, there is a relation between the speed of oxidation and the free energy of the system. As the E'_0 of the system becomes more positive, the time necessary to oxidize the reduced dye increases in a logarithmic function. It must once more be emphasized that this relation is not a general one. The case of 1-naphthol-2-sulfonate-indo-2,6-dichlorophenol has been found to be an exception, as the dye is oxidized at higher speed than expected. On the other hand, hydroquinone is oxidized at lower speed than predicted. But although there are many more exceptions which do not allow the formulation of a rule establishing a relation between chemical affinity and free energy when different systems are compared, these same exceptions when taken individually show the same relationship between the available free energy of the system and speed of oxidation. When the rate of oxidation is determined at frequent intervals, all the dyes which have been studied as well as hydroquinone show the same relationship: the free energy of the system as measured by the oxidation potential is a function of the logarithm of the time of oxidation, exactly as found when comparing different systems.

These observations seem to be of importance for an understanding of the problem of biological oxidations. The relation between the catalytic power of reversible dyes upon cellular oxygen consumption and their E'_0, which was reported some time ago and has been the origin of these studies, becomes clear, since the oxidation of the same dyes in homogeneous solutions shows the identical relation. The same applies to the relation between the speed of reduction of dyes by tissues and their oxidation-reduction potential. The natural catalyst for cell oxidations seems to be, according to War-

burg (18), a hemin compound, where the oxidations are performed through a change of valence of the iron nucleus. Here, too, it is possible that oxidations are performed according to the potentials of the hemin catalyst, since these potentials vary with the change of the nitrogen derivatives attached to the coordination places left free in the iron-pyrrole complex, as Conant and Tongberg (19) have shown. Once more it must be emphasized that the data given in this paper are only in the nature of a contribution to a further study on this controversial subject.

SUMMARY

The autoxidation by molecular oxygen of a number of reversible oxidation-reduction dyes has been studied by means of an electrometric method. At constant pH and in the absence of catalysts it has been found that there is a linear relation between the E'_0 of the dye and the logarithm of the time necessary to oxidize the dye from 2 per cent to 50 per cent oxidation. This is not a general rule, as 1-naphthol-2-sulfonate-indo-2,6-dichlorophenol is oxidized at greater speed. On the other hand hydroquinone is oxidized at lower speed than predicted if the rule was general.

When the E'_0 of the system is changed by changing the pH of the solvent, the same relation between E'_0 and speed of oxidation has been found.

When the rate of oxidation of single oxidation-reduction systems is studied, the identical relation exists between the oxidation potential of the system and the rate of oxidation. In such a case even those systems which did not oxidize at the predicted time, show this relationship.

The rate of oxidation of some of the dyes can be expressed by the equation of reactions of the first order.

The significance of these findings and its supposed contradiction to well known thermodynamic assumptions is discussed.

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THE EFFECT OF ADMINISTRATION OF IODINE ON THE TOTAL IODINE, INORGANIC IODINE, AND THYROXINE CONTENT OF THE PATHOLOGICAL THYROID GLAND

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Recent chemical investigations (1) have made it possible to partition the iodine compounds known to be present in the thyroid gland. It was thought that application of this advance could be made to a study of the distribution of iodine in the thyroid glands of patients with hyperthyroidism after iodine had been administered. An insight into the chemical changes taking place in the diseased thyroid gland after ingestion of iodine, which might thus be obtained, would contribute to our knowledge of the function of the gland, both normal and pathological. In this communication, the results of the estimation of total iodine, inorganic iodine, and thyroxine in a series of thyroid glands are presented.

The iodized pathological gland contains iodine in organic and inorganic combination (2, 3): as the iodized amino acids, *thyroxine* (4) and *diiodotyrosine* (5, 6), all presumably incorporated in the thyroglobulin molecule; and as *inorganic iodides* in varying amount, depending on the dosage and duration of iodine administration and on the type of gland. These comprise, so far as we know, all the iodine compounds in the gland. For separation of the total iodine of the pathological thyroid gland into these component iodine fractions, we made use of the following scheme.

Total iodine

(Aqueous extraction of desiccated
gland)

Inorganic I-   -Organic I

(Alkaline hydrolysis)

Thyroxine I | Diiodotyrosine I

Total iodine, inorganic iodine, and thyroxine iodine were determined directly, while total organic iodine was derived by difference. From the data thus obtained, it is possible to study the composition of the thyroglobulin contained in the pathological glands examined, by calculating the thyroxine iodine fraction of total organic iodine.

$$\frac{\text{Thyroxine I}}{\text{Total I} - \text{inorganic I}} = \frac{\text{thyroxine I}}{\text{organic I}} \backsimeq \frac{\text{thyroxine I}}{\text{thyroglobulin I}}$$

The assumption that practically all the organic iodine present is incorporated in the thyroglobulin molecule (7) is based on the results of the dialysis (8) and proteolytic digestion (9) of thyroid gland preparations.

EXPERIMENTAL

The material investigated by this procedure consisted of 137 thyroid glands: 117 pathological glands obtained after subtotal thyroidectomy, and twenty normal glands. The clinical history and thyroid histology relating to each case were studied. The fresh surgical specimen was weighed, a small piece removed for histological section, the remaining portion weighed again, and after mincing with scissors, dried in a Freas oven at 70–80° until suitable for grinding with mortar and pestle (usually 24 hours, rarely up to 72 hours). The pulverized gland was again dried in the oven at 60° for 24 hours, and brought to constant weight *in vacuo* over $CaCl_2$. The water content was calculated by difference. If sufficient material was available, 4 gm. of the dried powder were defatted with petroleum ether for 10 hours in a Soxhlet apparatus, the fat content being determined by direct weighing of the fat residue. The defatted gland was again brought to constant weight *in vacuo*.

The *total iodine content* was determined in duplicate on 0.05 to 0.25 gm. samples by a slightly modified Kendall method (10). *Inorganic iodine* was estimated by aqueous extraction of the desiccated, non-defatted gland in duplicate samples of 0.1 to 1.0 gm., according to the material available. The samples were weighed into 50 cc. narrow neck centrifuge tubes, and 30 cc. of iodine-free water and 1 drop of caprylic alcohol added. The tubes were tightly stoppered and vigorously shaken in a shaking machine for 2 hours at room temperature. After centrifuging ½ hour at 2000 R.P.M., the supernatant fluid was decanted into a nickel dish, the residue mixed with 10 cc. of water, centrifuged again, and the clear supernatant liquid added to the first portion in the nickel dish. 5 cc. of 10 N NaOH were added, the contents of the dish evaporated almost to dryness, ashed, and the iodine content determined in the usual manner.

This method of estimation of the iodide content is based on the old observation (11, 12) that, although most of the thyroglobulin can be extracted by water from fresh glands, only traces go into aqueous solution from the denatured *desiccated* gland. The method used is essentially that of Harington and Randall (13). Aqueous extraction of the dry gland does not give a *complete* separation of inorganic from organic iodine (no means of effecting a quantitative separation is known (14, 15)), but the following evidence is presented, which suggests that the separation is adequate for our purpose. Table I shows the amount of iodine extracted by 30 cc. of water from desiccated glands in 2 hours, the amount of iodine obtained from the residue by a second 2 hour extraction, and the iodine removed by a third extraction of the second residue. The recovery of added KI suggests the adequacy of aqueous extraction of inorganic iodide within 2 hours.

We found that 95 to 98 per cent of the iodine in the aqueous extract was ultrafiltrable. The extract was filtered with suction through a collodion membrane made in a Coors porcelain filter of 50 cc. capacity, following a suggestion of Dr. F. E. Kendall. Silver precipitation of the aqueous extract (10) was carried out on several of the glands in which aqueous extraction indicated a high iodide content. In all such glands, over 90 per cent of the iodine extracted by water gave an acid-insoluble silver precipitate. The silver method could not be used to check results on glands

containing less than 0.1 mg. of inorganic iodine in the sample used, as recoveries from known KI solutions of this concentration were low. Representative experiments, suggesting that over 90 per cent of the iodine extracted from desiccated glands is in inorganic form, are summarized in Table II.

TABLE I

Aqueous Extraction of Desiccated Thyroid Gland with and without Added KI

| Gland No. | Size of sample | I₂ added | I₂ extracted | | | Added I₂ recovered in 2 hrs. |
			First 2 hrs.	Second 2 hrs.	Third 2 hrs.	
	gm.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
1	0.2	0	0.317	0.0021	0.0015	
	0.3	0	0.468	0.0059	0.0032	
2	0.5	0	0.095	0.0029	0.0027	
	0.5	0.05	0.145	0.0058	0.0035	0.050
	0.5	0.10	0.200	0.0077	0.0029	0.105
3	0.2	0	0.376	0.0091		
	0.1	0.05	0.240			0.052
	0.1	0.10	0.291			0.103
	0.1	0.20	0.390			0.202

TABLE II

Results of Silver Precipitation and of Ultrafiltration of Aqueous Extracts

| Gland No. | I₂ in aqueous extract | | |
	By direct ashing	Iodide as I₂ by Ag method	Non-ultrafiltrable
	per cent	*per cent*	*per cent*
1	0.157	0.146	0.0051
3	0.184	0.175	0.0035
4	0.143	0.135	0.0028

A slight turbidity of the aqueous extract of the non-defatted gland, which persists despite prolonged centrifuging, is due to lipid, as shown by its disappearance when shaken with ether or when defatted glands are used. The turbidity may also be removed by adding 5 cc. of aluminum gel (Willstätter's Type C) before the 2 hours shaking. The iodine content of the water-clear extract remains unchanged, so there is no need of removing the suspension. The aqueous extract, even when made water-

clear, must be ashed, as direct titration gives an unsatisfactory end-point.

Other methods of separation of inorganic from organic iodine were investigated: extraction with alcohol, acetone; precipitation with phosphotungstic acid, trichloroacetic acid, silver sulfate; and ultrafiltration. Aqueous extraction of the desiccated gland was found to give the most complete recovery of added KI, and has the added advantages of simplicity and availability of the residue for further analysis, if the gland is small.

Thyroxine iodine was determined in duplicate on 0.5 to 1.25 gm. samples by the method of Leland and Foster (1). The great discrepancy between the results obtained by their method and those obtained by acid precipitation after 4 hours hydrolysis (13, 16) was pointed out by Leland and Foster, who found in the same material a thyroxine iodine of 23 per cent of the total iodine by their procedure and 48 per cent by the acid-insoluble fraction method. We obtained similar results. This discrepancy largely disappears, however, if acid precipitation is carried out after hydrolyzing for 24 hours in aqueous alkaline solution (17). Thus a sample of thyroid containing 25 per cent of the total iodine as thyroxine iodine by the Leland and Foster method, gave 41 per cent by the acid-insoluble fraction method after 4 hours hydrolysis with normal NaOH, but only 27 per cent by the same method after 24 hours hydrolysis. The acid-insoluble fraction in a series of determinations gave a strong biuret reaction at 4 hours, which was absent only after 12 to 16 hours hydrolysis. Kendall, who introduced the acid-insoluble method for the determination of thyroxine, later obtained evidence that not all the iodine in acid-insoluble form is thyroxine (2, 18). Our observations support Foster's suggestion that the acid-insoluble fraction method, as hitherto used, gives high values because hydrolysis is incomplete in 4 hours, and acidification at the end of that period throws down not only such thyroxine as has been liberated, but a heterogeneous group of polypeptides still containing diiodotyrosine as well as thyroxine. The decrease in acid-insoluble iodine when hydrolysis is continued over 4 hours cannot be accounted for entirely by destruction of thyroxine by alkali, as has been assumed, because Leland and Foster obtained increasing amounts of iodine in their butyl alcohol fraction as hydrolysis with 2 N NaOH was

prolonged from 4 to 18 hours; and because more than 90 per cent of thyroxine which has been boiled 24 hours with normal NaOH can be recovered (1, 2). As the determination of thyroxine by the acid-insoluble fraction method is indirect, and even after 24 hours hydrolysis gave poor agreement between duplicates in our hands, we chose the Leland and Foster method. We determined thyroxine in dry defatted glands and corrected for the inorganic iodine in the thyroxine titration figures when such correction lowered the thyroxine iodine value 5 per cent or more. It is also possible to determine thyroxine in the residue left after aqueous extraction. In our series of thyroxine determinations on 137 glands, the mean discrepancy between duplicates was 2.8 per cent.

TABLE III

Determination of Thyroxine I in 1 Gm. of Dry Thyroid after Addition of KI

Added KI	I₂ in BuOH	Increase in I₂ in BuOH	
		Calculated (added I₂ × 0.168)	Found
mg. I₂	*mg.*	*mg.*	*mg.*
0	0.642	0	0
10	0.815	0.168	0.173
20	0.974	0.336	0.332
20	0.997	0.336	0.355
40	1.321	0.672	0.679

No correction was attempted for the loss due to destruction during hydrolysis, which may be up to 15 per cent (1).

In applying the method to iodized pathological glands, the following points of difference between such material and non-iodized normal glands were considered.

1. The great increase in inorganic iodine in the gland when iodides were administered before thyroidectomy might interfere with the determination of thyroxine. Experiments in which comparatively large amounts of KI were added to desiccated thyroid before hydrolysis showed good agreement between the amount found in the BuOH fraction and that calculated on the basis of 1.68 per cent (1) of the iodine added as KI. Table III suggests that even when iodine as KI is present in an amount more than 60 times the thyroxine iodine content of the gland (in our

most extreme case, exophthalmic Gland 26, the ratio was 40:1) the thyroxine iodine may be calculated if the inorganic iodine is known. At the same time, the necessity for determining inorganic iodine in such material is illustrated.

2. Some pathological glands contain greatly diminished thyroxine contents. We found the method adequate, as far as agreement between duplicates was concerned, for glands with thyroxine iodine contents as low as 0.001 per cent.

3. In some instances, the amount of material available did not permit the use of duplicate samples of 1.25 gm. which Leland and Foster found to give highest thyroxine yields. We obtained good agreement with 0.50, 0.75, and 1.25 gm. samples, and in order further to check the analyses, chose samples of different weights throughout.

4. It has been suggested that intermediate organic iodine compounds, as yet unidentified, might exist in the pathological gland (19, 20). Such compounds might conceivably pass into the butyl alcohol fraction. On subjecting one possible intermediate compound, diiodothyronine,[1] to the Leland and Foster method of extraction, only 17.7 per cent of this substance (so closely related structurally to thyroxine) appeared in the butyl alcohol extract.

<div align="center">DISCUSSION</div>

Our results on the different types of glands examined are summarized in Tables IV to VIII. Only those obtained with iodized exophthalmic glands[2] will be discussed in detail here, because essentially the same chemical changes take place after iodine administration in both types of toxic glands, but the picture in adenomatous glands may be complicated by the admixture of large amounts of connective tissue. In Table IV we present our data on 65 iodized exophthalmic glands arranged in order of increasing total iodine content. The significance of these results is best brought out by comparison with those on normal glands from

[1] β-3,5-Diiodo-4-(4'-oxyphenoxy)-phenyl-α-aminopropionic acid obtained from Hoffmann-LaRoche, Inc., through the courtesy of Dr. F. Gudernatsch.

[2] Thyroid glands obtained from patients with exophthalmic goiter who had received a preoperative course of iodine are referred to in this paper as iodized exophthalmic glands.

TABLE IV

Iodine Partition of Thyroid Glands: Exophthalmic Goiter

Gland No.	Basal metabolic rate above normal		Estimated weight of whole dry gland	Iodine per gm. dry gland			Thyroxine I per cent of total organic I
	Pre-iodine	Post-iodine		Total	Thyroxine	Inorganic	
	per cent	*per cent*	*gm.*	*mg.*	*mg.*	*mg.*	
1	30	−6	11.0*	0.68	0.030	0.15	5.7
2	49	30	18.2	0.74	0.021	0.24	4.2
3		−8	9.6*	0.75	0.029	0.19	5.2
4	16		6.9	0.76	0.064	0.25	12.6
5			5.4*	0.80	0.140	0.18	22.6
6	63	24	9.8*	0.90	0.070	0.29	11.5
7	51	6	7.2	0.91	0.182	0.11	22.7
8	58	20	14.4	0.93	0.068	0.36	11.9
9	28	3	12.1	0.96	0.075	0.33	11.9
10	34	9	11.2	1.00	0.032	0.49	6.3
11	65	37	18.0	1.01	0.111	0.26	14.8
12	68	63	11.3	1.12	0.076	0.18	8.1
13		35	5.4*	1.12	0.038	0.72	9.5
14	52	8	9.1	1.23	0.058	0.51	8.1
15		19	14.5	1.26	0.067	0.69	11.8
16	55	11	11.3	1.29	0.143	0.31	14.6
17	48	12	8.9	1.35	0.122	0.45	13.6
18	41	15	3.8	1.38	0.047	0.76	7.6
19	42	25	13.0	1.45	0.258	0.16	20.0
20	50	37	23.2	1.51	0.123	0.63	14.0
21	64	63	7.4*	1.54	0.160	0.30	12.9
22	26	3	7.5	1.60	0.086	0.42	7.3
23	33	18	10.6	1.64	0.261	0.27	19.0
24	40	17	3.7	1.68	0.253	0.64	24.0
25	64	32	11.5	1.79	0.063	0.96	7.6
26	72	22	9.3	1.91	0.033	1.43	6.9
27	89	44	11.7*	1.98	0.239	0.63	17.7
28	48	39	7.1	2.03	0.083	1.11	9.0
29		33	9.4	2.10	0.485	0.21	25.7
30	38	0	7.7	2.12	0.276	0.31	15.3
31	62	19	10.0	2.12	0.210	0.58	13.6
32	77	86	10.1	2.14	0.535	0.15	26.9
33		53	9.4*	2.15	0.348	0.68	23.7
34	43	17	19.1	2.17	0.071	1.43	9.5
35		43	6.0*	2.19	0.214	0.35	11.6
36	55	20	4.8*	2.26	0.302	0.71	19.4
37	49	33	10.7	2.27	0.403	0.36	21.1
38		42	8.2*	2.37	0.369	0.61	21.0

TABLE IV—*Concluded*

Gland No.	Basal metabolic rate above normal		Estimated weight of whole dry gland	Iodine per gm. dry gland			Thyroxine I per cent of total organic I
	Pre-iodine	Post-iodine		Total	Thyroxine	Inorganic	
	per cent	*per cent*	*gm.*	*mg.*	*mg.*	*mg.*	
39	54	2	15.4	2.44	0.458	0.36	22.0
40	45	30	8.2	2.54	0.566	0.35	25.8
41	28	24	12.3	2.57	0.340	0.84	19.7
42	68	35	17.8	2.57	0.548	0.36	24.8
43	45	27	11.8	2.69	0.166	1.41	13.0
44	78	14	5.1*	2.69	0.508	0.56	24.3
45	69	49	10.2	2.69	0.643	0.25	26.3
46	55	23	15.2	2.80	0.540	0.70	25.7
47	28	31	2.9*	2.84	0.493	0.95	25.9
48	33	20		2.84	0.544	0.88	27.5
49		42	8.9*	2.96	0.333	0.50	13.4
50	85	44	8.2*	3.15	0.329	1.36	18.4
51	69	37	8.9	3.17	0.497	0.75	20.3
52	64	27	5.8	3.24	0.618	0.77	25.0
53	60	36		3.33	0.679	0.56	24.5
54		38	7.1	3.37	0.678	0.70	25.4
55	86	34	17.2	3.46	0.763	0.41	25.0
56	35	28	4.8	3.60	0.824	0.42	25.9
57	74	60	21.4	3.88	0.695	1.14	25.2
58	47	42	10.6	3.90	1.100	0.43	31.7
59	58	29	15.2	4.05	0.874	0.59	25.3
60	65	30	11.6	4.15	0.481	1.57	18.6
61	38	32	20.6	4.20	0.397	1.85	16.9
62	68	14	19.5	4.53	1.033	0.44	25.3
63	38	17	7.0	4.65	0.795	0.31	18.3
64	45	18	7.9	6.55	1.315	0.75	22.6
65		33	11.5	6.71	1.230	0.92	21.2

The weight of the whole gland was estimated by measuring with calipers the thyroid remnant left *in situ*, cutting out a corresponding piece from the excised gland, and adding the weight of the piece thus obtained to the weight of the whole excised portion. The amount of gland left *in situ* was found to vary with the size of the gland consistently enough to permit calculation of the fragment left behind from the weight of the excised portion. The error involved in estimating the weight of the whole gland is probably within ±10 per cent.

* One lobe only.

this vicinity and with such data on non-iodized exophthalmic glands as were found in the literature. Because of the variability observed, comparison was made not only of maxima, minima, and averages, but also of the frequency distribution of the several groups studied. We included the results of analysis of thyroglobulin of five iodized exophthalmic glands reported in Table VI to make up a group of 70 iodized exophthalmic glands and compared this group with the results on 70 normal glands. The normal series comprised the data on 50 normal glands reported by Leland and Foster and our results on twenty glands.

The *total iodine content* of 65 iodized exophthalmic glands, in mg. per gm. of dry gland, varied from 0.68 to 6.71, averaging 2.35, which exceeds the average total iodine content of 70 normal glands (1.86) and is much greater than that of non-iodized exophthalmic glands (0.26, average of forty-four glands, calculated from Wilson and Kendall (21); 0.59, calculated from three glands, Lunde et al. (22); 0.75, calculated average of an unstated number of glands, Kocher (23)). A frequency distribution study of our series of iodized exophthalmic glands showed some spread both above and below the range of normal glands with normal histological structure. The spread above probably has no further significance than to indicate iodine storage and is determined chiefly by the amount and duration of iodine administration. More striking is the fact that 15 per cent of our pathological glands contain less than 1 mg. of iodine, the lower limit compatible with normal thyroid structure (24), even after iodine administration in a type of gland known to absorb iodine with avidity.

Comparison of the average total iodine content of iodized with non-iodized exophthalmic glands suggests that administration of iodine to patients with exophthalmic goiter may result in an increased iodine content of the thyroid from the low values of non-iodized glands up to or above the normal range.

The *total iodine content of the whole gland*, in mg., in 57 iodized exophthalmic glands varied from 5.2 to 88.3, averaging 28.2 mg., compared with an average of 8.85 mg. for 70 normal glands, and of 5.1 and 5.87 for non-iodized exophthalmic glands (calculated from data reported by Wilson and Kendall (21) and Kocher (23)). An increased iodine content of exophthalmic glands following administration of iodine is again indicated.

Partition of the iodine compounds in the gland after iodine intake enables us to go a step further and show how the increase in total iodine indicated by the above data is distributed among the iodine fractions in the gland. The *inorganic iodine content* in 65 exophthalmic glands, in mg. per gm. of dry gland, varied from 0.11 to 1.85, averaging 0.59; considerably greater than the average inorganic iodine content (0.13) of twenty normal glands. No data on the iodide content of non-iodized exophthalmic glands could be found in the literature, but it is presumably low. The *inorganic iodine content of the whole gland*, in mg., in 57 iodized exophthalmic glands, varied from 0.8 to 38.1, with an average of 7.5, as compared with the average of 0.58 in twenty normal glands. Administration of iodine to patients with exophthalmic goiter, therefore, results in a marked increase in the inorganic iodine content of the gland. Consideration of the *per cent of total iodine in inorganic form* in 65 iodized exophthalmic glands indicated that between 6.7 per cent and 74.9 per cent of the increase in total iodine following iodine intake in our series was due to absorption of iodides by the gland. The per cent of total iodine in inorganic form in any one gland probably undergoes constant change.

The *thyroxine iodine content* of 70 exophthalmic glands, in mg. per gm. of dry gland, varied from 0.021 to 1.315, averaging 0.37, somewhat lower than the average thyroxine iodine content of 70 normal glands (0.474), but much greater than the average of eleven non-iodized exophthalmic glands (0.042 mg.) calculated from Wilson and Kendall who used the acid-insoluble fraction method, which gives high results.[3] Comparison of the frequency distribution of iodized exophthalmic glands with that of normal glands indicates that more than 25 per cent of the pathological glands have thyroxine iodine contents between 0.02 and 0.10 mg. per gm., the latter the lower limit compatible with normal histological structure. Yet the greater average thyroxine iodine content of iodized exophthalmic glands, as compared with the average of non-iodized exophthalmic glands (computed from the data of

[3] Kendall's acid-insoluble fraction iodine was about 50 per cent of the total iodine in normal glands (8) under the conditions of hydrolysis employed here (21). This is about twice the thyroxine iodine per cent of total iodine obtained by the method of Leland and Foster in normal glands.

Wilson and Kendall), suggests an increase in the thyroxine content of the gland following administration of iodine. In a large number of glands, the thyroxine iodine content, although apparently increased following administration of iodine, is still very low as compared with normal glands.

The *thyroxine iodine content of the whole gland*, in mg., of 57 exophthalmic glands, varied from 0.2 to 20.1, averaging 4.4, which is greater than our figure for 70 normal glands (2.24), and much greater than that calculated from Kendall's data for non-iodized exophthalmic glands (0.82 mg., average of eleven glands, by the acid-insoluble fraction method, calculated from Wilson and Kendall). These figures again suggest an increase in the thyroxine content of the exophthalmic gland after administration of iodine.

The *thyroxine iodine per cent of total organic iodine* is of particular interest, as it affords an insight into the chemical structure of the thyroglobulin molecule in different stages of thyroid disease and remission. 70 iodized exophthalmic glands varied from 4.2 to 31.7 per cent, averaging 17.5 per cent, which is greater than the average of eleven non-iodized exophthalmic glands (16 per cent, Wilson and Kendall, from data obtained by the acid-insoluble fraction method, which gives high values), but is much lower than the average of 70 normal glands (25.5). The distribution of iodized exophthalmic glands with respect to their thyroxine iodine per cent of total organic iodine, when compared with the distribution in an equal number of normal glands, shows a large number of exophthalmic glands below the normal range. Indeed, practically all our pathological glands lie below the average normal value. In exophthalmic glands in which the concentration of thyroglobulin iodine is low, we find in most cases, a concomitant change in the composition of the thyroglobulin, as shown by a decrease in its thyroxine content. Fig. 1, in which the ratio of thyroxine iodine to total organic iodine is plotted against total organic iodine for our 70 iodized exophthalmic glands, suggests that with the increase in thyroglobulin iodine after iodine administration, there is an increase in the thyroxine complement of thyroglobulin up to, but not above, the normal ratio. Although in several of our glands the administration of iodine resulted in an increase in thyroglobulin iodine well above the maximum of non-iodized normal glands, in no instance did the
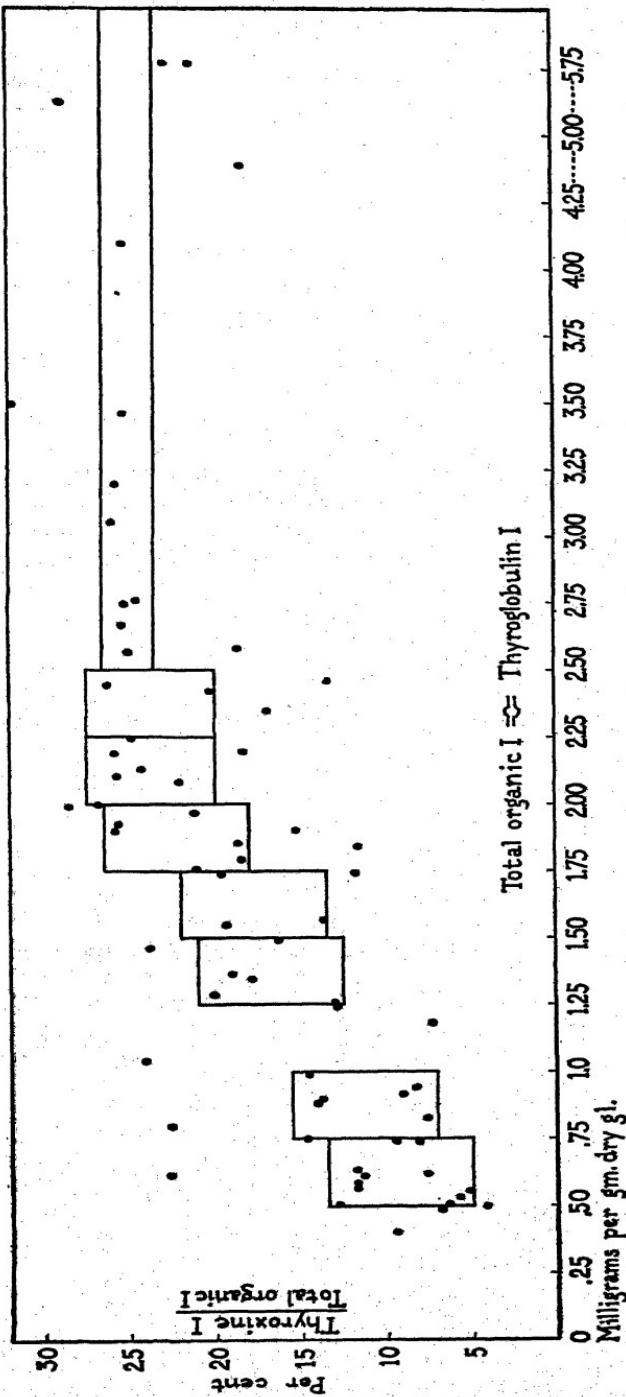
Fig. 1. Scatter diagram to show the relationship between increasing thyroglobulin iodine to total organic iodine in our series of 70 iodized exophthalmic glands. The trend is indicated by lines drawn to include areas containing the majority of points.

thyroxine iodine per cent of total organic iodine exceed the normal maximum of about 35 per cent. The low thyroxine complement of thyroglobulin found in non-iodized and in some iodized exophthalmic glands is thought to be due to a relative increase of a thyroxine-poor thyroglobulin. The increase in the thyroxine iodine per cent of thyroglobulin iodine following iodine administration may result from a relative increase of thyroxine-rich thyroglobulin, or thyroglobulin with its full complement of thyroxine.

TABLE V

Iodine Partition of Thyroid Glands: Adenoma with Hyperthyroidism

Gland No.	Basal metabolic rate above normal		Estimated weight of whole dry gland	Iodine per gm. dry gland			Thyroxine I per cent of total organic I
	Pre-iodine	Post-iodine		Total	Thyroxine	Inorganic	
	per cent	*per cent*	*gm.*	*mg.*	*mg.*	*mg.*	
1	27	9	38.0	0.22	0.017	0.017	8.4
2	22	28	8.8	0.32	0.019	0.035	6.7
3	15	?	14.2	0.70	0.099	0.094	16.8
4	28	17	9.7	0.98	0.171	0.180	21.4
5	73	40	71.9	0.99	0.071	0.210	9.1
6	62	43	11.5	1.42	0.198	0.260	17.1
7	33	?	11.0	1.56	0.297	0.192	21.9
8	73	40	11.3	1.57	0.190	0.289	14.8
9	33	?	63.4	1.63	0.370	0.196	25.9
10	35	13	24.1	1.98	0.246	0.189	14.0
11	50	30	17.3	1.99	0.405	0.158	22.4
12	42	29	8.9	2.03	0.399	0.221	22.1
13	27	28	24.4	2.76	0.553	0.445	23.8
14	52	36	12.3	2.88	0.636	0.217	23.9
15	30	21	21.4	3.13	0.500	0.262	17.4
16	16	2	5.9	3.35	0.773	0.160	24.2
17	62	52	10.6	5.50	1.150	0.613	23.6
18	24	4	6.3	5.96	1.196	0.476	21.8

The results obtained on eighteen adenomas with hyperthyroidism are summarized in Table V. The data suggest changes in the glands of the same character as those noted above in exophthalmic glands after administration of iodine. We wish merely to point out that in this type of gland, the total iodine and thyroxine iodine content may remain much lower than in exophthalmic

glands.   We believe this is related to the increase in fibrous tissue and to the morphological type of adenoma.

In Table VI, the results on thirteen thyroglobulin preparations obtained by one of us several years ago (W. W. P. (25)) from iodized exophthalmic glands and iodized adenomas with hyperthyroidism are summarized.   The data are in agreement with those derived from whole gland preparations.

We present in Table VII a summary of the results obtained on twenty-one simple adenomas, removed from patients who did not

TABLE VI

Iodine Partition of Thyroglobulin Preparations

Prep-ara-tion No.	Diagnosis	Basal metabolic rate above normal		Total I	Thy-roxine I	Thy-roxine I per cent of total I
		Pre-iodine	Post-iodine			
		per cent	*per cent*	*mg. per gm.*	*mg. per gm.*	
1	Adenoma with hyperthyroidism	18	?	1.26	0.26	20.8
2	Exophthalmic goiter	38	16	1.47	0.24	16.3
3	" "	65	15	1.73	0.20	11.7
4	" "	47	17	1.85	0.34	18.6
5	Adenoma with hyperthyroidism	21	?	2.16	0.50	23.1
6	Exophthalmic goiter	53	29	2.19	0.40	18.3
7	Adenoma with hyperthyroidism	31	34	2.80	0.63	22.6
8	Exophthalmic goiter	12	13	3.18	0.47	14.7
9	" "	74	14	3.57	0.75	21.0
10	" "	79	29	5.47	1.50	27.5
11	Adenoma with hyperthyroidism	25	21	5.51	1.65	29.9
12				5.59	1.40	24.9
13	Adenoma with hyperthyroidism	19	8	5.79	1.71	29.5

receive a preoperative course of iodine, except the patient from whom Gland 20 was obtained.   Some of these patients received iodine before admission to the hospital.   We note many glands with low total iodine (23), low thyroxine iodine, and with a definite decrease in the thyroxine iodine per cent of total organic iodine, possibly explained in part by the increase in adventitious tissue.   Comparison with myxedematous and cretin glands would be instructive.

In Table VIII, we present our data on twenty glands obtained

from apparently normal individuals who had met with sudden traumatic death. The histological picture of these presumably normal glands was disturbingly variable (26). Inorganic iodides were found in every gland. How much was preformed, particularly in those cases which received surgical attention, and how much was split off in the process of desiccation, we do not know.

TABLE VII

Iodine Partition of Thyroid Glands: Adenoma without Hyperthyroidism

Gland No.	Estimated weight of whole dry gland	Iodine per gm. dry gland			Thyroxine I per cent of total organic I
		Total	Thyroxine	Inorganic	
	gm.	*mg.*	*mg.*	*mg.*	
1	16.7	0.08	0.005	0.004	5.9
2	17.4	0.10	0.011	0.041	18.3
3	8.5	0.23	0.009	0.023	4.4
4	21.9	0.25	0.032	0.014	13.3
5	26.5	0.35	0.047	0.018	14.2
6	14.3	0.40	0.045	0.025	12.0
7	43.0	0.54	0.074	0.068	15.7
8	37.0	0.57	0.041	0.170	10.3
9	42.3	0.58	0.052	0.063	10.0
10		0.62	0.038	0.076	7.0
11	10.3	0.64	0.085	0.132	16.0
12	23.0	0.77	0.132	0.070	9.1
13	6.1	0.82	0.229	0.039	29.5
14	21.8	0.97	0.177	0.043	19.4
15	46.0	1.00	0.147	0.086	16.2
16	11.5	1.47	0.278	0.119	20.7
17	28.5	1.56	0.358	0.063	24.0
18	11.6	1.79	0.401	0.047	23.0
19	8.3	2.02	0.191	0.413	11.9
20	9.2	2.53	0.561	0.211	24.1
21	8.4	3.76	0.940	0.432	28.2

Our results are in general agreement with those of Leland and Foster (1).

The changes from the normal content and distribution of iodine, which are found in the typical exophthalmic gland, and the effect of iodine administration upon these changes are brought out in Fig. 2. Comparison of the average normal gland (A) with the average non-iodized exophthalmic gland (B), calculated from the

data of Wilson and Kendall, suggests that, as a result of disease, the following changes have taken place: (1) a decrease in the total iodine content (23, 27), (2) a decrease in the thyroxine iodine content (19, 21), (3) a decrease in the percentage of thyroglobulin iodine as thyroxine iodine (21), (4) an increase in the percentage of

TABLE VIII

Iodine Partition of Thyroid Glands of Normal Individuals

Gland No.	Dry weight	Iodine per gm. dry gland			Thyroxine I per cent of total organic I	Histology
		Total	Thyrox-ine	Inor-ganic		
	gm.	*mg.*	*mg.*	*mg.*		
1	6.21	0.57	0.11	0.06	21.6	Early involution
2	3.51	0.84	0.20	0.10	27.0	Normal, connective tissue increased
3	3.74	1.07	0.22	0.07	22.0	Normal
4	4.24	1.16	0.26	0.05	23.4	" early involution
5	3.74	1.32	0.17	0.07	13.6	Early hyperplasia
6	9.13	1.56	0.30	0.08	20.3	Normal, connective tissue increased
7	4.27	1.65	0.26	0.08	16.6	Early involution, baso-philic colloid
8	2.70	1.70	0.24	0.07	14.7	" "
9	7.93	1.71	0.43	0.10	26.7	Normal resting gland
10	3.02	1.73	0.40	0.32	28.4	" " "
11	3.08	1.82	0.57	0.11	33.2	Early involution
12	8.22	1.85	0.50	0.07	28.1	Normal resting gland
13	8.88	2.28	0.49	0.13	22.8	"
14	5.18	2.28	0.62	0.10	28.4	"
15	6.78	2.55	0.65	0.25	28.3	Normal resting gland
16	3.97	2.72	0.90	0.10	34.3	" " "
17	3.34	2.82	0.97	0.09	35.5	" " "
18	4.98	2.83	0.67	0.32	26.7	"
19	4.05	3.86	1.21	0.18	32.9	" resting gland
20	4.08	4.31	1.38	0.20	33.6	" " "

thyroglobulin iodine not in the form of thyroxine but as diiodo-tyrosine (+ some as yet unidentified organic iodine compound (?)), (5) a decrease in the inorganic iodine fraction (?).

While considerable quantities of iodine and colloid may be found in thyroid glands associated with clinical manifestations of severe thyrotoxicosis (see Table IV; *cf.* Oswald (27), Tobler (28), Jackson
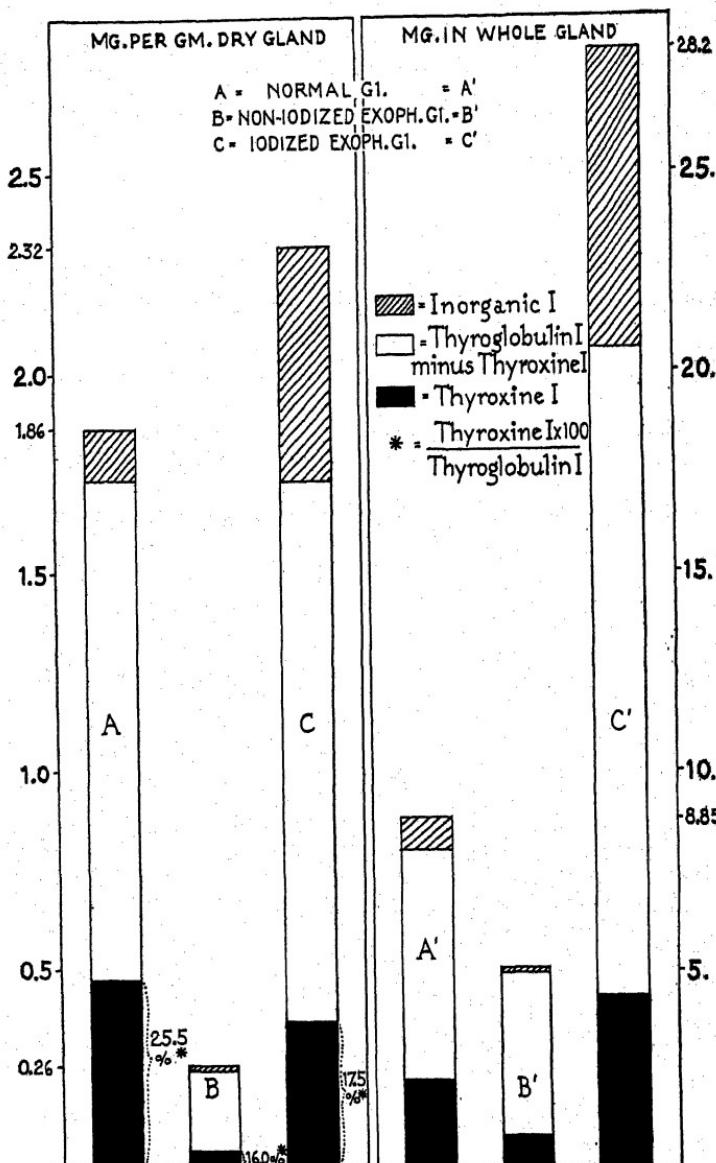
FIG. 2. The average total iodine, thyroxine iodine, and inorganic iodine of 70 normal, 44 non-iodized, and 70 iodized exophthalmic glands are compared in terms of mg. per gm. of dry gland and mg. in the whole gland. A is based on data of Leland and Foster and our own; B is calculated from Wilson and Kendall.

and Ewell (29)), the typical non-iodized exophthalmic gland is more or less depleted of its iodine compounds. Clinical (30), histological (31), and micro respiratory studies on excised tissue (32) and the isolated gland (33) suggest that this depletion is not due to a decrease in secretory activity of the gland but to an increase in the liberation of its hormone into the blood. An increased rate of diffusion of thyroid hormone into the circulating fluid could result in the decrease in the total and thyroxine iodine content of the gland which we have described. The decreased thyroxine iodine per cent of thyroglobulin iodine may be similarly explained if, as has been suggested (34), diiodotyrosine is a precursor in the synthesis of thyroxine in the thyroid gland. Since such evidence as we have suggests that thyroxine is formed relatively slowly (35, 36), it seems not improbable that too rapid discharge of the gland content would eventually result in a relative increase of thyroglobulin without its full complement of thyroxine. The data do not indicate whether alteration in the normal regulatory mechanism of storage and discharge of hormone is due to overstimulation of the gland, to permeability changes, or to a combination of these factors. The possibility of a cooperating hormone is not excluded.

In Fig. 2, comparison of non-iodized (B) with iodized exophthalmic glands (C) emphasizes the changes following administration of iodine suggested by frequency distribution studies already described. An increase in the total iodine content of hyperplastic thyroid glands following iodine administration has been repeatedly demonstrated (35, 37, 38). That exophthalmic glands, showing increasing degrees of regression histologically, contain increasing amounts of iodine in acid-insoluble form, was pointed out by Wilson and Kendall (21). Weir obtained higher results by the same method (39), and attributed the increase to iodine administration to his patients. The iodine content of thyroglobulin is greater in the iodized than in the non-iodized exophthalmic gland (23). Lunde and Wülfert (3) found the iodine content of thyroglobulin of iodized exophthalmic glands lower than that of normal glands, but in two cases where iodine had been given for longer periods, the thyroglobulin iodine was normal. Both Kocher (23) and Lunde and Wülfert (3) observed an increase in the inorganic iodine content of the gland.

It appears, then, that the data in the literature, derived by quite different methods, are in general agreement as to the increase in total iodine and the general nature of its distribution in the thyroid gland of patients who have received iodine. Broadly speaking, the non-iodized exophthalmic gland, enlarged, but depleted of its iodine compounds, has been converted into a gland which, gm. for gm., cannot be distinguished from a normal gland, except for its increased inorganic iodine content; but because of its enlargement, now contains much greater absolute amounts of total iodine and the constituent iodine compounds.

It seems probable that both clinical remission and the change from the depleted state toward the state of repletion characteristic of the normal resting gland are secondary to, and dependent upon a slowing up of the accelerated liberation of thyroid hormone into the circulating fluids. Direct evidence for this retardation has been obtained by estimation of the organic iodine fraction of the blood (40), which is markedly increased in exophthalmic goiter, but falls toward normal levels after administration of iodine to the patient. From the data at present available, it seems unlikely that an incompletely iodized hormone elicits the toxic symptoms encountered in hyperthyroidism.

With excretion into the circulating fluids retarded, but secretory activity of the gland marked for at least a limited period after the administration of iodine was begun (19), an increase in the organic iodine content of the gland, such as we found, is to be expected. The increase in the thyroxine iodine per cent of thyroglobulin iodine may simply be due in part to the retention of thyroglobulin in the gland long enough to afford the time necessary for thyroxine synthesis to take place. The increase in the inorganic iodine content of the gland is undoubtedly due to absorption from the blood. It would appear, therefore, that the effects of iodine on the exophthalmic gland can be explained by the absorption of inorganic iodine from the blood, and the storage of organic iodine in the gland. It seems likely that these two processes are themselves closely related (20), and that in further chemical study of their relationship may lie the approach to a more adequate comprehension of clinical and physiological phenomena associated with thyroid function.

A discussion of the significance of the chemical studies reported

here in relation to the clinical picture and course of hyperthyroidism will be the subject of a later communication.

SUMMARY

1. A procedure for the partition of the known iodine compounds of the thyroid gland is described, based on the method of Leland and Foster for the determination of thyroxine, a slightly modified Kendall method for determination of total iodine, and aqueous extraction of the desiccated gland for estimation of inorganic iodine. The data derived by this procedure on 117 pathological thyroid glands obtained after thyroidectomy, and on twenty normal glands are presented.

2. Comparison of the results on 70 thyroid glands of patients with exophthalmic goiter who had received iodine, with the data in the literature on thyroid glands of untreated patients with exophthalmic goiter suggests the following changes as a result of iodine administration: (1) an increase in the total iodine content of the gland, due to an increase in both the inorganic iodine and thyroglobulin iodine fractions; (2) a change in the chemical nature of the thyroglobulin as evidenced by (a) an increase in the thyroxine iodine per cent of thyroglobulin iodine and (b) a consequent decrease in the per cent of thyroglobulin iodine not in the form of thyroxine, but chiefly or entirely as diiodotyrosine; (3) an increase in the relative and absolute thyroxine and diiodotyrosine content of the gland.

3. Comparison with the data on normal glands suggests that these changes constitute a return from the more or less depleted state of the untreated exophthalmic gland toward that of the resting gland.

4. The physiological significance of the changes in the content and distribution of iodine in the pathological thyroid gland is briefly discussed.

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ISOLATION AND IDENTIFICATION OF VITAMIN C*

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The previous work reported from this laboratory (1–5) on the chemical nature of vitamin C has been continued, particularly in the study of the solubility of the active material in organic solvents, leading towards its isolation by crystallization. This paper deals with (a) the precipitation of the active material as the lead salt, and (b) the isolation of a crystalline compound which is active in preventing scurvy in guinea pigs. The properties of this active crystalline substance correspond with those given for the "hexuronic acid" studied by Szent-Györgyi (6–7) as an oxidation-reduction factor in adrenal cortex, oranges, and cabbage. We believe that the two substances are identical, as stated in a previous communication (8).

EXPERIMENTAL

The method used for testing the activity of our preparations was essentially that described by Sherman, La Mer, and Campbell (9), except that we have adopted a shorter time interval (56 days) to facilitate fractionation studies. The procedure employed for concentrating the antiscorbutic factor was essentially the same as that previously reported from this laboratory (4, 5).

2.5 liters of lemon juice were decitrated and an absolute acetone solution of the active material was obtained according to the general method previously reported. This solution was evaporated to dryness and taken up in 10 cc. of n-propyl alcohol. To this was added an equal volume of petroleum ether. The solid, previously found to be of low activity (1–5), was centrifuged off and the clear liquid divided into two parts. A half saturated solution of lead acetate in absolute methyl alcohol was added to one part

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TABLE I

Precipitation by Lead and by Petroleum Ether

Test preparation	No. of animals	Fed daily, lemon juice equivalent	Average gain or loss in weight	Average survival (56 day test)	Average scurvy score
		cc.	*gm.*	*days*	
First lead precipitate............	5	10–5*	171	56	2
Second " "	5	10–5*	–107	49	13
Solids not precipitated by lead....	4	10–5*	–100	35	16
Combination of above three fractions...........................	4	10–5*	149	56	1
Ethyl acetate-petroleum ether-soluble fraction...................	4	10–5*	–111	35	14
Ethyl acetate-petroleum ether-soluble and insoluble fractions....	5	10–5*	80	56	3
Ethyl acetate before adding petroleum ether.....................	5	10–5*	153	56	2
Positive controls................	2	1–2†	103	56	1
Negative "	3	0	–95	24	13

* The lemon juice equivalent was reduced from 10 cc. to 5 cc. on the 31st day.

† The lemon juice was increased from 1 cc. to 2 cc. on the 40th day.

TABLE II

Recrystallization of Vitamin C

Test preparation	No. of animals	Fed daily, lemon juice equivalent	Average gain or loss in weight	Average survival (56 day test)	Average scurvy score
		cc.	*gm.*	*days*	
Residue in solvent after					
First crystallization.................	2	10	–103	29	15
Second "	5	10	–109	33	18
Third "	5	10	–125	30	17
Recrystallized solid....................	5	10	86	56	3
Material less soluble in ethyl acetate...	1	10	–30	56	7
	4	10	–101	43	10
Combination of soluble and crystallized solids....................	3	10	122	56	1
Positive controls....................	3	1	96	44*	1
Negative "	3	0	–131	27	19

* Started 12 days late on test.

of the solution until precipitation was somewhat over half completed. The yellow semicrystalline solid was centrifuged and dissolved in alcoholic hydrochloric acid to remove the lead. This was made up in aqueous solution, after evaporating off the hydrochloric acid, and stored in an ice box under carbon dioxide for feeding. Precipitation in the centrifuged liquid was then completed by the addition of more lead solution, and the second precipitate was treated in the same manner as the first. The results given in Table I indicated that the active material was precipitated by lead, with most of the activity in the first precipitate.

FIG. 1. Vitamin C crystallized from butyl alcohol and petroleum ether, magnified about 250×, photographed with polarized light.

The remainder of the clear liquid from the petroleum ether-propyl alcohol precipitation was evaporated to dryness and extracted with anhydrous ethyl acetate overnight in an ice box. Purified quartz sand was mixed with the residue to facilitate extraction. The ethyl acetate extract was evaporated to approximately 5 cc. and an equal volume of petroleum ether added. A light yellow semicrystalline material was thrown down which, on standing at room temperature, formed a sirup. From Table I is it evident that the greater part of the active material was in the fraction which separated from solution. By carrying out the precipitation in dry ice, a pale yellow crystalline material was obtained. This

was centrifuged and redissolved in ethyl acetate. It was found that all of the material would not redissolve in a small volume of ethyl acetate, but, instead, a part remained as a semisolid which, when dried in a vacuum desiccator, formed needle-like crystals resembling the lactone form of the hexuronic acid described by Szent-Györgyi. The material soluble in ethyl acetate was again quickly precipitated in dry ice by the addition of petroleum ether and again dissolved in ethyl acetate. A third crystallization was carried out at room temperature, the petroleum ether being added slowly. When the solution was allowed to stand overnight in the ice box, crystals formed on the side of the flask. The activity of this preparation is indicated by the data given in Table II. During the animal tests no attempt was made to build up distinct crystals, but, instead, preparations were made rapidly in dry ice to provide a fresh preparation each week for feeding. Each feeding level accordingly represents the results of from eight to ten preparations.

It will be seen from Table II that the final crystalline solid which was readily soluble in ethyl acetate and the material which was less soluble were both active. The difference shown in the activity of the two materials is apparently due to the difference in quantity of total solids fed. The more soluble substance gave a solution containing approximately 0.05 mg. per cc. of lemon juice equivalent, compared to 0.03 mg. for the less soluble substance. This would place the activity of the first crystalline preparation fed on a level of 0.5 mg. per day, compared to 0.3 mg. of the lactone. The difference in animal response for the two groups indicates that the protective value of the two substances was practically equivalent when considered on a weight basis.

The vitamin can be recrystallized readily from butyl alcohol (Fig. 1), acetone, ethyl acetate, ethyl alcohol, or methyl alcohol, by the addition of petroleum ether. The appearance of the crystals varies with different solvents.

DISCUSSION

The material which we have described previously in our vitamin C concentrates has shown a protective antiscorbutic activity in the general range of 0.5 to 1.0 mg. of solids per guinea pig per day. The lower figure represents the approximate protective level of

our most active preparations during the past year's work involving several series of tests. The properties observed, both chemical and physical, have strongly indicated that the active factor was similar to the hexuronic acid described by Szent-Györgyi and studied as a reducing agent in animal and plant tissues. We have completed several fractionating treatments with only slight fluctuations in activity, and there is practically no change in activity when the material is (a) crystallized rapidly from solution in mixed solvents or a series of solvents, (b) precipitated as a lead salt from water or alcohol, or (c) converted to a lactone form of different solubility.

Reprecipitation with lead from aqueous or alcoholic solution can be repeated consistently. It seems unlikely that a crystalline material would retain the same activity through repeated recrystallizations with different solvents. The activity of the lactone form, differing markedly in solubility, also points strongly toward the antiscorbutic factor's being identical with the crystalline hexuronic acid. The purification of this substance from adrenal cortex has also been recently reported by Kendall (10) who has kindly submitted a sample of hexuronic acid for comparative studies. We will report our results on testing this independent preparation in the near future. It is active.

We believe that there is no serious disagreement between the above conclusion regarding the chemical nature of vitamin C and experimental data reported from other laboratories. According to a recent paper by Rygh and coworkers (11), the normal course of scurvy is disturbed by feeding an o-diphenol derivative of narcotine. The close structural relation of the phenolic portion of this compound to adrenalin is striking, and it may be that the adrenal function was disturbed in these experiments, which in turn was manifested by a disturbance of the vitamin C function. The high concentration of the hexuronic acid in adrenal cortex indicates a close relationship to the glandular function, in addition to its more general rôle as (a) a reactant in plant and animal oxidation-reduction systems, and (b) its antiscorbutic activity. The experimental results and conclusions of Rygh et al. could not be verified by Smith and Zilva (12), nor could we interpret their experimental findings as indicating that the o-diphenol derivative of narcotine could

function as vitamin C.[1]   Animals supplied with the compound failed to survive longer than those receiving a vitamin C-free diet. The animals which were fed an aqueous extract of sprouted grain and those which received 5 cc. of heated orange juice probably received all of their vitamin C from these sources, for it has been clearly indicated from previous studies that both of these supplements would have supplied an appreciable amount of the protective factor.

In earlier studies on electrical transference, clear evidence was obtained of the acidic character of the active factor, and there was no evidence of a compound containing basic nitrogen groups being involved.   For over a year, our purest preparations have given negative phenolic reactions with ferric chloride.   To consider the vitamin only a specific factor in relation to *typical* hemorrhages and bone abnormalities, would, in our opinion, require a new definition or conception of the vitamin.   Its protection of *normal health* when the animal is on a vitamin C-free diet is a more adequate test of the presence of the vitamin.   For that reason, we have always used the general animal response as a guide, in addition to a conventional scoring system.

The oxidation studies on vitamin C by Zilva (13) are difficult to interpret at present, but it appears plausible that the results obtained with complex mixtures cannot be clearly interpreted in terms of analogy to a single compound.   If the partially oxidized product from vitamin C formed by indophenols can be reduced to the active factor in the animal body, whereas the aerobically oxidized product cannot, then Zilva's results in this respect could be clearly interpreted.

The evidence from which we conclude that vitamin C as isolated in our laboratory is identical with the hexuronic acid studied by Szent-Györgyi and Kendall as a reducing factor in plant and animal tissues, is as follows (*i.e.* they correspond in): (*a*) natural occurrence so far as studied (the protective level of 0.5 mg. daily of our preparation corresponds with 0.5 mg. of hexuronic acid estimated by Szent-Györgyi (1928) in 2 cc. of orange juice), (*b*) oxidation by iodine and by Benedict's reagent (quantitative), (*c*) optical

[1] Private communications from three different laboratories during the past few weeks have also indicated a failure to confirm Rygh and coworker's conclusion regarding the chemical nature of vitamin C.

rotation ($[\alpha]_D^{20} = + 25° \pm 1°$), (d) acid titration equivalent (exact for the free acid), (e) C and H combustion, for $C_6H_8O_6$, (f) reversible formation of a lactone and a free acid, (g) typical crystal forms, (h) solubility in a number of organic solvents, (i) precipitation as a lead salt, (j) instability toward alkalies and oxidizing agents, (k) diffusion rate and electrical transference (McKinnis and King (3)), (l) melting point (183–185°).

Additional studies on the chemical nature of Szent-Györgyi's hexuronic acid and our own crystalline vitamin C preparations are under way. In a preliminary paper which has just been received (14), it is evident that Szent-Györgyi and Svirbely have found the hexuronic acid (from adrenal glands) protective against scurvy on a 1 mg. level.

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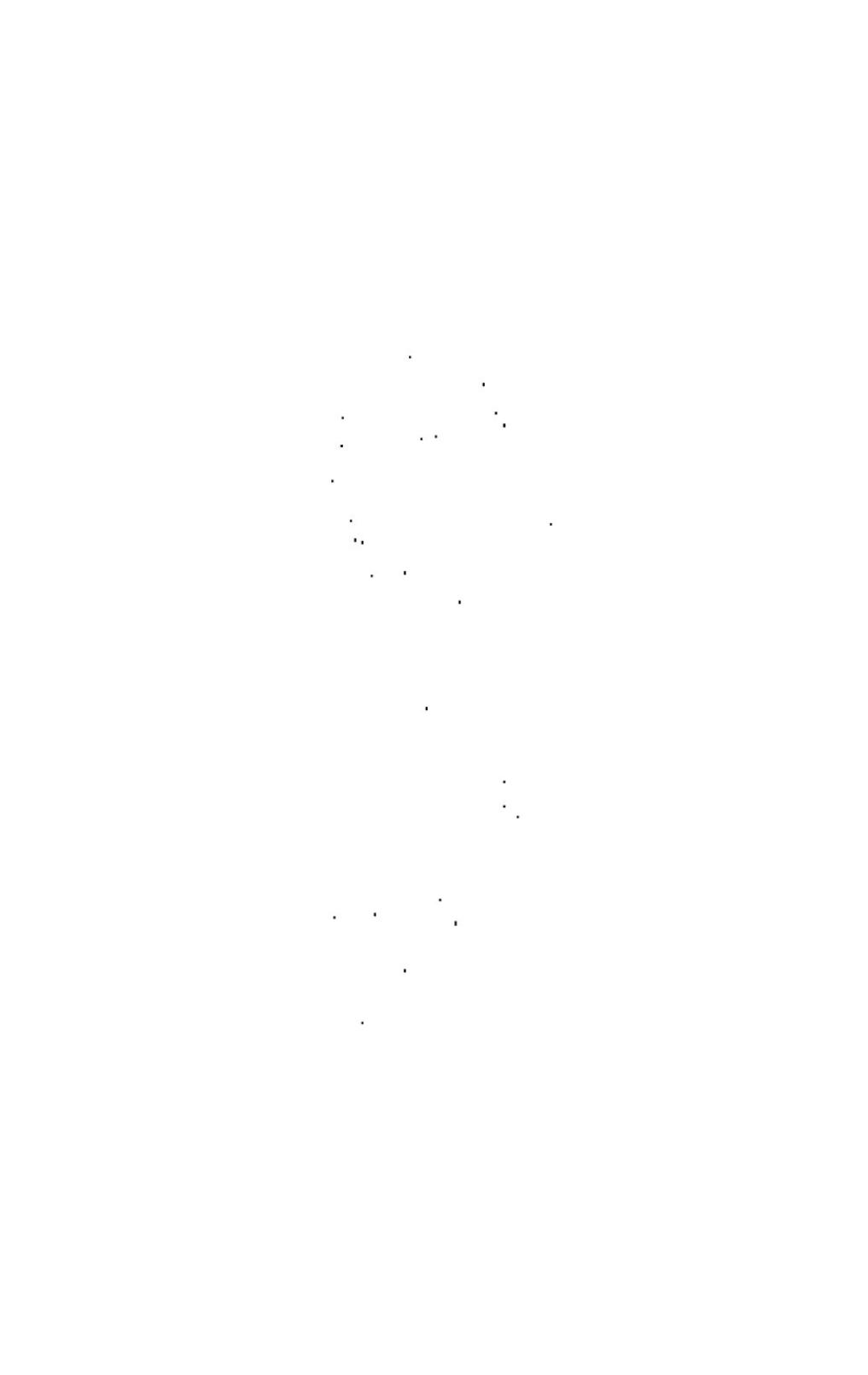
THE ISOLATION OF NORLEUCINE, WITH EVIDENCE FOR ITS IDENTITY, AND SOME THERMODYNAMIC DATA BASED ON THE DISSOCIATION PRESSURES OF THE COMPOUNDS WHICH THE ISOMERIC LEUCINES FORM WITH AMMONIA AND HYDROGEN CHLORIDE*

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In a recent monograph dealing with the history of the discovery of the naturally occurring amino acids, Vickery and Schmidt (1) have laid down certain criteria for the recognition of an amino acid as a definite product of the hydrolysis of proteins. On this basis a considerable number of substances which have been described as products of the hydrolytic cleavage of proteins was not included in the list of the accepted amino acids. Among these is norleucine, which was first described by Thudichum (2) and later investigated in somewhat greater detail by Abderhalden and Weil (3). In this connection, Vickery and Schmidt (1) state: "In view of the difficulty of separating the two known isomeric leucines by this method [i.e. fractional crystallization], not to mention the difficulty of removing traces of valine, which might have important effects upon the physical properties of the resulting product, it would seem only conservative to require still more convincing evidence of the existence of norleucine among the products of hydrolysis of proteins than that given by Abderhalden and Weil." It is the purpose of this paper to add further evidence as to the existence of norleucine as a product of the hydrolysis of certain proteins.

Method of Isolation

5 kilos of beef spinal cord were hydrolyzed with sulfuric acid in the usual manner and a crude amino acid fraction containing the isomeric leucines, valine and tyrosine, was obtained by direct
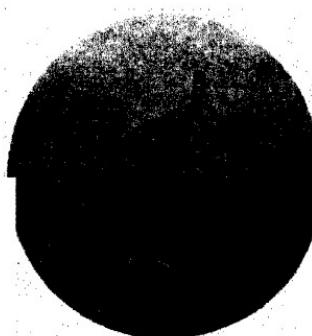
Fig. 1

Fig. 2   Fig. 3

Fig. 1. Photomicrograph[1] of norleucine crystals isolated from beef spinal cord protein.

Fig. 2. Photomicrograph of the copper salt of natural norleucine.

Fig. 3. Photomicrograph of the silver salt of natural norleucine.

crystallization. Further purification of this material was carried out by fractionation first of the copper salts and then of the zinc salts according to the technique which has been described by

[1] The photomicrographs were kindly taken for us by Miss Mary MacIntyre.

Brazier (4). The leucines were freed from tyrosine by solution in hot glacial acetic acid according to the method of Habermann and Ehrenfeld (5). The total nitrogen and the amino nitrogen corresponded to that of leucine; the crystal forms indicated that several leucines were present. The isomeric leucines were fractionated by differential solubility in water. Ten fractionations of the more insoluble portion which had a tendency to float on top of the solution were made. The crystal structure of the tenth fraction was identical with that of synthetic norleucine which was prepared by treating α-brom-n-caproic acid with ammonia. Photomicrographs of the natural norleucine, as well as the copper and silver salts, are shown in Figs. 1 to 3. The two latter products were identical with similar compounds which were prepared from synthetic norleucine. Analysis of the final product gave the following values: total nitrogen 10.70 per cent, amino nitrogen 10.69 per cent. The natural product is dextrorotatory. $[\alpha]_D = 22.5°$ at 25° when the material is dissolved in 20 per cent hydrochloric acid. The yield was about 11 gm. Due to large losses, this probably represents only a small portion of the norleucine content of the spinal cord proteins.

In order to determine the homogeneity of our norleucine preparation, its identity with synthetic α-amino-n-caproic acid and the relative amounts of leucine and norleucine which were present in our various crystal fractions, we have prepared titration curves of the solid materials with gaseous ammonia. The principle underlying this method is that the dissociation pressure of any given compound at any particular temperature is a constant. When the dissociation pressures of compounds such as those which are formed when monoaminomonocarboxylic acids are treated with gaseous ammonia or hydrogen chloride are considerable, such pressure measurements can be used to characterize individual amino acids or to identify each of the components when contained in a mixture of several amino acids.

The apparatus which we have used is essentially the same as that which Bancroft and his coworkers (6) employed in their phase rule studies of the proteins and other compounds. It consists of a closed system with a manometer, a reaction flask which can be shaken, and an attached burette for measuring the volume of gas which has been added or withdrawn. A side tube was added for

the purpose of admitting water vapor.   It was found essential that a small amount of water vapor be present in order that the reaction might proceed.   The water probably acts as a catalyst.   The amount which was added was not sufficient to constitute a separate component of sufficient magnitude to warrant consideration. In order that the amount of water vapor which was added might be definitely known, the amino acids which were titrated were previously finely ground with carbon dioxide snow, passed through a 100 mesh screen, and dried for several weeks in a high vacuum over phosphorus pentoxide.

The amino acid .or mixture of amino acids was placed in the reaction flask, the entire system was evacuated to a definite pressure, usually 1 mm., and a definite amount of water vapor, usually also 1 mm., was added.   Since the partial pressures of these two gases are additive, the total or 2 mm. was subtracted from the ammonia dissociation pressure of the compound. For the purpose of these studies the amino acids were titrated with pure dry ammonia gas.   It was added in convenient amounts to the amino acid, the reaction flask was shaken, and, when equilibrium was established, the dissociation pressure was measured.   The entire apparatus was kept in an air bath at $25° \pm 0.25°$.

In such a system containing one amino acid there are two components, the amino acid and the ammonia.   There are three phases, the gas phase and the two solid phases, the amino acid and the ammonia salt of the amino acid.   According to the phase rule $(F + p = C + 2)$ there is but one degree of freedom.   When at any particular temperature the amount of ammonia (in cc.) is plotted against its partial pressure, the curve will be a straight line until a small quantity of ammonia has been added beyond that required to form the ammonium salt of the amino acid.   The pressure of ammonia will then rise.   When more than one amino acid is present, the curve will not only show the partial pressure of each compound which forms an ammonium salt, but also, on the basis of the amount of ammonia which is required for the titration, the amount of each of the several amino acids which may be present in the mixture.   The method is especially useful in characterizing individual amino acids since direct comparison between a synthetic and a naturally occurring amino acid can be made by measurement of the dissociation pressure.   A mixture of the synthetic and the

natural amino acid should show no break in the curve when titrated
with ammonia. The presence, however, of another amino acid
would be indicated by a break in the straight line curve.

In these experiments it was not found necessary to correct for
the fugacity of ammonia since, at the pressures employed, it
behaves as a perfect gas. Corrections for the vapor pressure and
the thermal expansion of mercury are unnecessary since, at the
temperatures employed, they are within the limits of error inherent
in the method. The time necessary for equilibrium to be reached
after each addition of ammonia gas depends somewhat on the
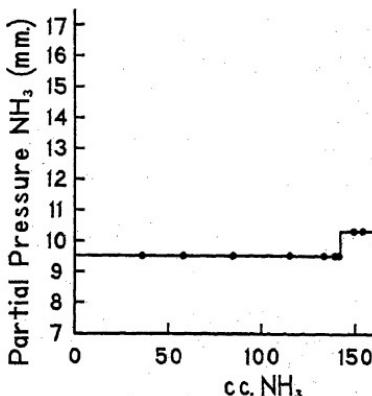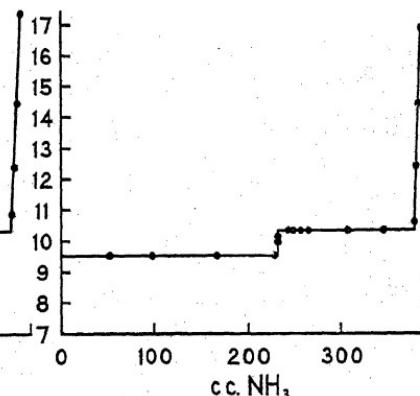
FIG. 4. Ammonia titration and dissociation curve of a mixture of 0.75
gm. of dry leucine and 0.25 gm. of dry norleucine.

FIG. 5. Ammonia titration and dissociation curve of the fourth dry
crystal fraction obtained from spinal cord protein hydrolysate. The
crystals are a mixture of leucine and norleucine, the former predominating.

amount of ammonia already added. Equilibrium was always
reached within a period of 2 days.

The leucine titration data are graphically represented in Figs.
4 to 7. Fig. 4 shows the titration curve of a mixture containing
0.75 gm. of leucine and 0.25 gm. of norleucine. The first portion of
the curve represents not only the dissociation pressure of the
ammonia-leucine compound, but also the amount of ammonia
required to complete the titration. When the requirements for
the formation of this compound have been completed, a break
occurs in the curve. The next portion indicates not only the

dissociation pressure of the ammonia-norleucine compound, but also the amount of ammonia required for the formation of the second compound. The next break in the curve indicates merely addition of excess ammonia. Fig. 5 shows the titration curve of the fourth crystal fraction which was obtained from the spinal cord hydrolysate. The dissociation pressures correspond to those of leucine and norleucine. The curve shows that the amount of leucine present in the mixture is approximately 1.5 times that of norleucine. The data obtained on titrating the seventh crystal fraction, which is graphically represented in Fig. 6, show that the
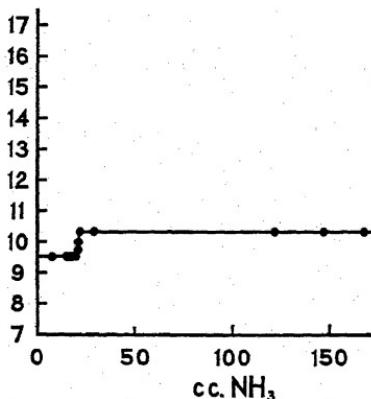
FIG. 6.
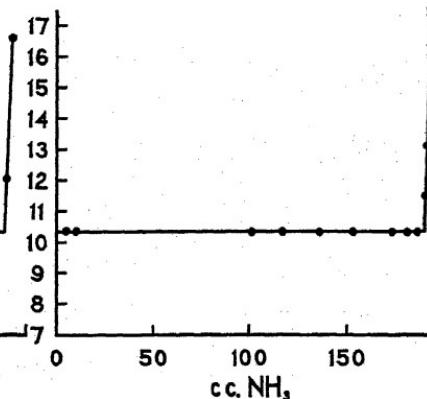
FIG. 7.

FIG. 6. Ammonia titration and dissociation curve of the seventh dry crystal fraction obtained from spinal cord protein hydrolysate. The crystals are a mixture of leucine and norleucine, the latter being in excess.

FIG. 7. Ammonia titration and dissociation curve of the tenth dry crystal fraction obtained from spinal cord protein hydrolysate. The curve indicates the presence of norleucine only.

mixture consists chiefly of norleucine, with a reduction of the leucine portion to about 10 per cent. The curve depicted in Fig. 7 is characteristic of norleucine and shows no leucine contaminant.

In Table I are given the ammonia and the hydrogen chloride dissociation pressures of the three isomeric leucines. The *d*-leucine is the product which was obtained from the spinal cord protein hydrolysate. The other compounds were preparations of known purity. Both the ammonia and the hydrogen chloride dissociation pressures of the natural and the synthetic norleucine

are identical, thus further characterizing our preparation as being norleucine. For convenience, we have introduced the term $pK_b{}^s$ to denote the dissociation pressure of a compound which is formed between a solid substance and a gaseous base, and the term $pK_a{}^s$ to denote the dissociation pressure of a compound which is formed between a solid substance and a gaseous acid. The terms $pK_a{}^s$ and $pK_b{}^s$ are defined as the negative (common) logarithms of the dissociation pressure or fugacity in atmospheres of a compound existing in the solid state. It is assumed, as will be shown later, that the free amino acid in the dry state exists as a *Zwitter Ion*. It will be noted from Table I that there is less difference between the $pK_a{}^s$ values of the three isomeric leucines than between the corresponding $pK_b{}^s$ values. The difference between the $pK_a{}^s$

TABLE I

	$pK_b{}^s$	$pK_a{}^s$
dl-Norleucine...................................	1.867	2.440
d-Norleucine....................................	1.866	2.441
dl-Isoleucine...................................	1.877	2.440
dl-Leucine......................................	1.900	2.450

The values for $pK_b{}^s$ are for the combination with ammonia. The values for $pK_a{}^s$ are for the combination with hydrogen chloride.

values would probably be greater if an acid weaker than hydrogen chloride had been used.

In order to calculate values for ΔF, ΔH, and ΔS we have determined the dissociation pressures of the compounds which the three isomeric leucines form respectively with ammonia and hydrogen chloride at 2.5°, 25°, and 40°. The change in free energy with dissociation, ΔF, is given by the equation

$$\Delta F = -RT \ln K = -1364.9 \log K \qquad (1)*$$

where K is the dissociation pressure in atmospheres. Since its value is small, the value of $\log K$ becomes negative with the result

* The meaning of the symbols used in Equations 1, 2, and 3 is the same as given by Lewis, G. N., and Randall, M., Thermodynamics and the free energy of chemical substances, New York and London (1923).

that the value for ΔF becomes positive in sign. The change in heat content due to dissociation, ΔH, is given by the equation

$$- \Delta H = \frac{4.5787 \; d \log K}{d\left(\dfrac{1}{T}\right)} \tag{2}$$

In calculating values for ΔH the assumption was made that the increment ΔH is constant over the temperature range at which

TABLE II

Thermodynamic Data for the Three Isomeric Leucines

	dl-Leucine	*d*-Nor-leucine	*dl*-Iso-leucine	Gas employed for titration
	mm.	*mm.*	*mm.*	
Dissociation pressure at 25°.......	2.70	2.75	2.73	HCl
	9.55	10.32	10.12	NH₃
" " " 40°.......	3.40	3.45	3.45	HCl
	16.95	18.40	17.55	NH₃
" " " 2.5°......	2.05	2.05	2.10	HCl
	3.85	4.00	3.95	NH₃
pK_b^s (25°)	1.90	1.86	1.88	
pK_a^s (25°)	2.45	2.44	2.44	
$\Delta H_{K_b^s}$ (*calories*)	7050	7140	6800	
$\Delta H_{K_a^s}$ "	2840	2820	2900	
$\Delta F_{K_b^s}$ "	2590	2550	2560	
$\Delta F_{K_a^s}$ "	3340	3330	3330	
$\Delta S_{K_b^s}$ (" *per degree*)........	14.96	15.39	14.22	
$\Delta S_{K_a^s}$ " " "	−1.68	−1.71	−1.44	
Apparent dissociation constants in aqueous solution, 25°				
pK′ₐ............................	9.60	9.76	9.68	
pK′_b............................	11.64	11.61	11.64	

determinations were carried out. That this assumption is approximately correct is shown by the fact that straight lines were obtained when $\log K$ was plotted against $\dfrac{1}{T}$. The entropy change of the system due to dissociation ΔS is given by the equation

$$\Delta F - \Delta H = - T\Delta S \tag{3}$$

The thermodynamic data are given in Table .II.

Our experiments incidentally point to the fact that the amino acids when in the dry state exist almost wholly as *Zwitter Ionen* (7). In the dry state the dissociation pressures of the ammonium salts of the amino acids are in the neighborhood of 10 mm. On the other hand, the dissociation pressures of the ammonium salts of the lower fatty acids, such as acetic acid and propionic acid, are in the neighborhood of 1 mm. If ammonia combines with the carboxyl group of a monoaminomonocarboxylic acid in the same way as it does in the case of a fatty acid, it is to be expected that the dissociation pressure would be considerably less than that which was found. Addition of ammonia then depresses the dissociation of the amino group, while addition of hydrogen chloride
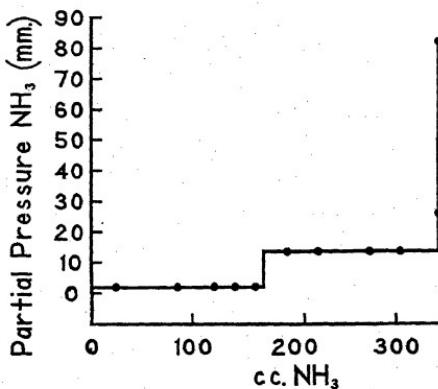
FIG. 8. Ammonia titration and dissociation curve of dry glutamic acid

depresses the dissociation of the carboxyl group. We may represent the *Zwitter Ion* form of the amino acid in the solid state by the schematic formula $(\overset{+}{NH_3}-R-\overset{-}{COO})_n$. The values for ΔF and ΔH, as shown in Table II, are much larger than the corresponding values which can be calculated for the ammonium salts of the fatty acids. On the assumption that the amino acids exist as *Zwitter Ionen*, these values are to be expected.

The distinction between a carboxyl group which exists as a *Zwitter Ion* and one which is free is shown by the ammonia titration curve of glutamic acid given in Fig. 8. The first portion of the curve shows a low ammonia dissociation pressure which is characteristic of a fatty acid. The second portion of the curve shows a

high dissociation pressure which is characteristic of the *Zwitter Ion* form of the amino acid. The amount of ammonia which is required to titrate each of the two groups is the same.

When ammonia or hydrogen chloride is added to an amino acid in the dry state, combination does not take place until a sufficiently high energy level has been reached to cause a hydrogen atom to migrate. This is accompanied by a shift of an electron to the positive group of the amino acid, leaving the compound electrically neutral. These energy levels, as seen from Table II, are quite high. We may represent the combination between the dry amino acid and gaseous ammonia and gaseous hydrogen chloride respectively by the following schematic equations.

$$(\overset{-}{NH_2}\cdot R\cdot C\overset{-}{O}O\ \overset{+}{NH_4})_n \xleftarrow{\ NH_3\ } (\overset{+}{NH_3}\cdot R\cdot C\overset{-}{O}O)_n \xrightarrow{\ HCl\ } (\overset{-}{Cl}\ \overset{+}{NH_3}\cdot R\cdot COOH)_n$$
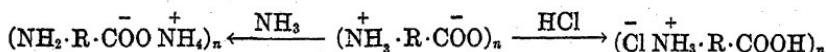
Work on the determination of the dissociation pressures of the compounds which other amino acids form with various gaseous acids and bases is under way.

SUMMARY

1. Norleucine has been isolated from spinal cord protein. By means of its crystal structure, the crystal structure of its copper and silver salts, and the ammonia and hydrogen chloride dissociation pressures, its identity with synthetic norleucine has been established.

2. The ammonia and hydrogen chloride dissociation pressures of the three isomeric leucines have been determined.

3. A method for determining the relative amounts of leucine and norleucine when present in a mixture of the two amino acids by titration with gaseous ammonia has been described.

4. Values for the change in free energy, the change in heat content, and the change in entropy due to dissociation of the ammonia and hydrogen chloride compounds of the three isomeric leucines have been determined.

5. It has been shown on the basis of the dissociation pressures and the thermodynamic data that the amino acids which have been studied exist when in the dry state as *Zwitter Ionen*.

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THE COPPER REDUCTION VALUES OF MANNOSE UNDER CERTAIN FIXED CONDITIONS*

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While performing a series of experiments on the intestinal absorption of hexose sugars (1), it became necessary to determine mannose dissolved in intestinal contents. A review of the literature revealed no satisfactory method, and no table expressing the copper-reducing power of mannose was found. Since we had successfully analyzed solutions of dextrose, galactose, and levulose by the Shaffer-Hartmann method as modified by Haskins (2, 3), we attempted the determination of mannose by the same method. As mannose is stereoisomeric with *d*-glucose, we believed that we could use the standard technique and establish a table expressing per cent of mannose in cc. of thiosulfate. Standard solutions of 0.5 to 2.0 per cent mannose were tested by the usual technique, and the results checked by three different investigators with uniformly poor results. The time allowed for reduction was varied from 7 to 30 minutes, and the time allowed for the reaction with sulfuric acid altered from 1 to 3 minutes. The determination for a given condition and quantity of sugar was checked by at least ten trials, with a variation of 2.0 to 5.2 cc. in the quantity of thiosulfate used. Because of some characteristic of mannose, even in pure solutions, it is impossible to get checks by this method.

Even though the quantitative determination by the phenylhydrazine precipitation method (4) may be accurate in pure solutions, it is inadvisable to use the method in the presence of foreign material. In intestinal fluids varying amounts of protein are carried down in the precipitate, which renders the weight

inaccurate. The copper reduction method of Bertrand (5) is unsatisfactory as the precipitate of cuprous oxide is also contaminated by varying amounts of protein which interfere with the subsequent titration with permanganate. The use of the polariscope is impractical on solutions of low percentage, and especially so when the sugar has as low a specific rotation as mannose (+14.25°). Small quantities of protein have been successfully removed by precipitation and filtration, but we have been unsuccessful in obtaining a protein-free filtrate of the unknown sugar solutions mixed with intestinal fluid.

A new method seemed necessary and after various experiments the following procedure was adopted as being satisfactory. The principle of the method, as used by us, is that of copper reduction. The reduced copper is separated by filtration and its weight determined either directly as cuprous oxide, or by the weight of the pure copper as determined by electrolysis. The cuprous oxide and copper equivalents of quantities of mannose in pure solutions, varying from 10 to 50 mg., were determined, and are presented in Table I.

Technique

For the purpose of description the procedure has been divided into three parts as follows:

To Determine Cuprous Oxide Equivalents of Mannose in Pure Solution—The mannose solution to be determined is measured accurately into a 100 cc. Pyrex beaker and a sufficient quantity of distilled water added to bring the total to 10 cc. 25 cc. of Benedict's qualitative reagent are added, and the beaker covered with a watch-glass and placed in a boiling water bath for 15 minutes. If the green color of the solution entirely disappears, it is an indication that too large a quantity of mannose is present and a smaller or more dilute sample should be taken. At the end of the 15 minute heating period the sample is rapidly cooled to room temperature in a water bath.

The precipitate is transferred quantitatively to a weighed, dry Gooch filter by filtering with suction. The beaker is rinsed three times with distilled water and cleaned with a rubber policeman, the rinsings being filtered through the same Gooch filter. The filter is dried overnight in a drying oven at 45° and allowed to cool

in a desiccator. As an alternative the precipitate may be washed and dried by the use of alcohol and ether washings, but if this method is used the Gooch filters must be treated in a similar manner before the preliminary weighing of them when empty. The filter and cuprous oxide are weighed, and the weight of the latter determined by subtraction. The weight of the actual copper can be calculated by multiplying the weight of cuprous oxide by

TABLE I

Cuprous Oxide and Copper Equivalents of Mannose

Mannose	Cuprous oxide	Copper	Mannose	Cuprous oxide	Copper
mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
10	27.7	25.6	31	75.7	67.3
11	30.0	26.6	32	78.0	69.3
12	32.4	28.8	33	80.2	71.3
13	34.7	30.8	34	82.5	73.3
14	37.1	32.9	35	84.7	75.3
15	39.4	35.0	36	87.0	77.3
16	41.8	37.2	37	89.2	79.3
17	44.1	39.2	38	91.5	81.3
18	46.5	41.4	39	93.7	93.3
19	48.8	43.5	40	96.0	85.3
20	51.2	45.5	41	97.9	87.0
21	53.4	47.5	42	99.9	88.7
22	55.6	49.4	43	101.8	90.6
23	57.8	51.4	44	103.8	92.3
24	60.1	53.4	45	105.7	94.1
25	62.3	56.3	46	107.7	95.7
26	64.5	57.4	47	109.6	97.5
27	66.8	59.4	48	111.6	99.2
28	69.0	61.4	49	113.5	100.8
29	71.2	63.3	50	115.5	102.5
30	73.5	63.3			

0.8882 (this figure is the quotient of the molecular weight of copper divided by that of cuprous oxide). This procedure was followed in arriving at the copper and cuprous oxide equivalents of the standard mannose samples, and the calculated weight of copper was checked by electrolysis as described in the following section.

Weight of Copper Determined by Electrolysis—The filter containing the cuprous oxide is placed in a 100 cc. Pyrex beaker and

25 cc. of dilute nitric and sulfuric acids added. The solution is accomplished by gently heating and stirring with a glass rod, care being taken to avoid spraying and loss of solution by overheating. The filter is washed as it is removed from the beaker to remove copper adherent to it. 50 cc. of an alkaline tartrate solution are added, and the copper determined by the electrolytic method of Peters (6) with a few modifications. Peters has devised a rapid electrolytic method for the determination of copper whereby the metal is deposited from an alkaline tartrate solution, such as is used in preparing Fehling's solution. The electrolysis is carried out either in platinum dishes placed upon plates of sheet brass to which the cathode connection is made, or in glass beakers or large test-tubes, in which case large cylindrical strips of sheet copper may be used for the cathode. We have employed 100 cc. Pyrex beakers with copper cathodes arranged to present the largest possible plating surface. The anode consists of a flat or cylindrical spiral of platinum foil or wire, which should not be placed at more than 1 cm. in distance from the cathode. We have devised an anode by fusing a piece of platinum foil 2.5 cm. square on the end of a piece of hollow glass tubing in such manner as to have a strip of the platinum project into the lumen of the tube. Contact with the source of direct current is made by introducing a small quantity of mercury into the tube in which the end of a wire may be placed. This gives a positive contact and allows the electrode to be placed at any depth in the solution without danger of bringing the copper wire in contact with the fluid. From 0.4 to 1.0 cc. of a saturated aqueous solution of potassium cyanide is then added, according to the amount of copper present; the amount of cyanide solution should be less than sufficient to discharge the blue color. If the copper deposit should be found to be too soft or dark colored, more cyanide should be used; an excess of the latter, however, greatly lengthens the time for complete deposition of the copper.

In making the determination the 110 volt direct current of a lighting system is used with three 32 candle power lamps interposed as resistance; under these conditions the voltage measures 2.6 and the amperage 2.85. We have used a small motor-driven generator which delivers essentially the same voltage and amperage. When more than two sets of electrodes are used, it is con-

venient to arrange a small rheostat between the source of energy and the electrodes to allow for adjustment. During electrolysis the solution is warmed by a small burner placed under the vessels. The evolution of gas and the circulation of warm liquid cause a very rapid deposition of copper, which is usually complete in 30 minutes for solutions of 40 mg. of copper or less. Longer intervals are necessary to plate according to the amount of copper present, the amperage used, the distance between electrodes, and the surface area of the electrodes.

To determine the completion of electrolysis, the Endemann-Prochazka (7) hydrobromic acid test is used. 1 volume of concentrated sulfuric acid is diluted with 2 to 3 volumes of distilled water. About 1 cc. of the dilute acid is placed in a narrow test-tube, a few crystals of potassium bromide are added, and the whole heated to boiling. A drop of the solution to be tested is then added; as small an amount as 0.007 mg. of copper will cause a red color to develop.

If the deposition of copper is complete, the solution in the cathode vessel, without breaking the current, is displaced by a small stream of water until the resistance lamps are extinguished; under this procedure no copper is lost by solution. When a small motor generator is used, the cathode is removed with the current constant and washed in water, alcohol, and ether, dried, and weighed.

Technique for Determination of Copper Reduced by Mannose Solutions Containing a Certain Amount of Protein—The same general method is used with the following changes. The weight of cuprous oxide is not determined, hence the Gooch filters used need not be dry or weighed, and no attempt is made to transfer quantitatively the cuprous oxide from the beaker to the filter, since the filtration is only necessary to eliminate the unreduced copper salt. A thorough washing of the precipitate and beaker is sufficient. The filter with its contained precipitate of cuprous oxide is placed in the Pyrex beaker with the remainder of the reduced copper and the estimation made by the same method as outlined above for the electrolytic determination of copper.

Pfanstiehl mannose, with a specific rotation of $+14.25°$ at $20°$, was used and all glassware checked against United States Bureau of Standards burettes. The method was found to be accurate

within the limits of 0.1 of 1.0 per cent for mannose in pure solutions. Rats were fasted for 48 hours before the introduction of mannose by stomach tube. The closed loops of dogs were washed, before the injection of mannose solutions, by the injection and aspiration of three subsequent quantities of 10 to 15 cc. of normal saline solution. In the recovery of the sugar from rats the intestinal fluid was diluted 1:150, that from the loops 1:50 (1). Under such circumstances the foreign material did not interfere with the accuracy of the method. The latter has been checked by the quantitative recovery of weighed amounts of mannose from fluid obtained from gastrointestinal tracts or loops when treated in the above manner.

Reagents

1. Benedict's Qualitative Reagent—100 gm. of anhydrous sodium carbonate and 173 gm. of sodium citrate are dissolved in 600 cc. of distilled water, with heat and continuous stirring. When completely dissolved, filter with cotton. Rinse the filter with distilled water. Add 17.3 gm. of pure copper sulfate dissolved in distilled water. Make the solution up to 1000 cc.

2. Dilute Acid Solution—This is prepared by the dilution of 65 cc. of concentrated sulfuric acid and 50 cc. of concentrated nitric acid to 1000 cc. with distilled water.

3. Alkaline Tartrate Solution—350 gm. of sodium potassium tartrate and 250 gm. of potassium hydroxide are dissolved in 500 cc. of distilled water with stirring and heat. A sufficient quantity of distilled water is added to total 1000 cc. of solution.

4. Saturated Aqueous Solutions of Potassium Cyanide—These are made up each week.

The acids, sodium potassium tartrate, potassium hydroxide, and potassium cyanide should be free of heavy metals.

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THE ISOLATION OF TRIMETHYLAMINE FROM SPORES OF TILLETIA LEVIS, THE STINKING SMUT OF WHEAT*

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Bunt, or stinking smut of wheat, is of common occurrence in most regions where wheat is grown extensively. Infection by the causal fungus takes place while the wheat plant is in the seedling condition; subsequently, the fungous hyphæ permeate the growing plant and eventually invade the young kernels, converting them into masses of dark brown spores. In the process of threshing, many of these infected kernels, or smut balls, are broken open and the spores which they contain are dusted over the surface of the sound grain. Wheat contaminated in this way is slightly dark in color and usually sells at an appreciable discount. The smut spores may also, although not always, impart to the grain a disagreeable fishy odor resembling herring brine. This odor is suggestive of trimethylamine, but, so far as the writers are aware, the isolation of this substance from bunt spores has not been accomplished. It is with this problem that the present paper deals.

The stinking smut disease may be caused by one of two fungi, *Tilletia levis* or *Tilletia tritici*. These two species, although closely related, may be distinguished from one another by the appearance of their spore walls, the spores of *Tilletia levis* being smooth, whereas those of *Tilletia tritici* are reticulate. In the

experience of one of the authors the production of the fishy odor already referred to is associated only with the spores of *Tilletia levis*. The two species have been grown on a number of wheat varieties, both in the greenhouse and in the field, and the spores of *Tilletia tritici* were invariably free from this odor. The experiments recorded in this paper, although somewhat incomplete, tend to confirm this observation.

Materials

The spores used in the present experiments were collected from wheat grown in 1931 on the plots of the Dominion Rust Research Laboratory at Winnipeg, Canada. One set of plots, comprising the varieties Little Club, Kota, Reward, and Ceres, was grown from seed which had been artificially inoculated with spores of *Tilletia levis*. Another set of plots of the same wheat varieties was grown from seed inoculated with spores of *Tilletia tritici*. Some additional collections of *Tilletia levis* were made from plots of Marquis, Marquillo, and Garnet wheat grown from artificially inoculated seed.

At harvest time a separate collection of infected heads was made from each plot. The smut balls were then removed from the heads and stored in paper bags in the laboratory until the end of February, 1932, when each lot was crushed in a mortar and the spores were sifted through a 200 mesh sieve. The material passing through the sieve consisted almost wholly of smut spores and represented approximately 75 per cent, by weight, of the smut balls.

Determinations were made of the total nitrogen in spores of *Tilletia levis* and *Tilletia tritici* collected on Ceres wheat. The spores of *Tilletia levis* were found to contain 4.51 per cent nitrogen and those of *Tilletia tritici* 4.41 per cent.

Determination of Total Volatile Base and of Ammonia

Preliminary experiments indicated that ammonia is evolved when an aqueous suspension of bunt spores is distilled under reduced pressure in the presence of barium hydroxide. It was therefore apparent that, if trimethylamine were the substance responsible for the characteristic odor of the spores, the amount recoverable from a given weight of spores could not be in excess of that repre-

sented by the difference between the total volatile base and the ammonia present in the distillate. With this consideration in view determinations were made of the total base and of the ammonia obtained on distillation from an equal weight of each collection of spores. The following procedure was adopted.

10 gm. of spores, together with 100 cc. of distilled water and 10 cc. of N sulfuric acid, were placed in a 2 liter Claissen flask. The mixture was heated and kept boiling for 3 minutes, after which it was cooled rapidly under the tap; 150 cc. of ethyl alcohol and 70 cc. of saturated barium hydroxide were added to the flask, and the mixture was distilled at about 60° under reduced pressure. Distillation was continued for 35 minutes from the time boiling began, the distillate being collected in 20 cc. of 0.1 N hydrochloric acid to which had been added 2 drops of methyl red. The total volatile base obtained from the spores was then determined by titration of the distillate with 0.1 N sodium hydroxide.

The neutral distillate was acidified with a few drops of 0.1 N hydrochloric acid, and the alcohol and most of the water were removed from it by distillation. The residue was made up to 100 cc. The ammonia in portions of this solution was then determined by Nessler's method. That this method can be employed for the determination of ammonia in the presence of trimethylamine has been shown by Vickery and Pucher.[1]

The results of the determinations are summarized in Table I, each value being the mean of two experiments. Only small collections of *Tilletia levis* spores were made from Marquis, Marquillo, and Garnet wheat, consequently the quantity of these spores used for each determination had to be reduced to duplicate 5 gm. portions. For purposes of comparison similar determinations were made with spores of *Ustilago tritici*, the loose smut of wheat; these results have also been included in Table I. In the calculation of the data no allowance was made for the moisture content of the spores. However, spores of *Tilletia levis* and *Tilletia tritici* collected on Ceres wheat were dried to constant weight over concentrated sulfuric acid in a vacuum desiccator; the loss in weight was found to be 4.6 and 4.7 per cent respectively. It is probable, therefore, that, after having been stored in the laboratory for several months, all of the spore collections had reached

[1] Vickery, H. B., and Pucher, G. W., *J. Biol. Chem.*, **83**, 1 (1929).

moisture equilibrium, and a recalculation of the results on a moisture-free basis would not appreciably affect any conclusions which might be drawn from them.

The data indicate that two collections of smut spores belonging to the same species, and similar in appearance, but grown on different varieties of wheat, may yield widely different amounts of ammonia. It is therefore apparent that the host plant plays an important rôle in determining the chemical composition of the smut spores which develop upon it. There is also some indication that spores which develop on high quality wheats, rich in protein,

TABLE I

Volatile Bases Obtained from Spores of Tilletia levis, Tilletia tritici, and Ustilago tritici

Wheat variety	Smut		Weight of spores	Total volatile base N	Ammonia N	Difference
			gm.	mg.	mg.	mg.
Little Club	*Tilletia levis*		10	5.22	4.44	0.78
" "	"	*tritici*	10	4.72	4.58	0.14
Kota	"	*levis*	10	5.96	5.24	0.72
"	"	*tritici*	10	5.01	4.85	0.16
Reward	"	*levis*	10	12.20	11.74	0.46
"	"	*tritici*	10	11.74	11.88	
Ceres	"	*levis*	10	8.43	7.73	0.70
"	"	*tritici*	10	7.66	7.11	0.55
Marquis	"	*levis*	5	5.71	5.20	0.51
Marquillo	"	"	5	7.19	6.74	0.45
Garnet	"	"	5	3.74	3.54	0.20
Several	*Ustilago tritici*		5	2.02	1.92	0.10

have a higher ammonia content than do those that develop on low quality wheat.

The spores of *Tilletia levis* contain significantly greater amounts of total volatile base than those of *Tilletia tritici* growing on the same wheat variety. Furthermore, the difference between total base and ammonia tends to be higher in spores of *Tilletia levis* than in those of *Tilletia tritici*. It will be shown later that the spores of *Tilletia levis* actually contain small quantities of trimethylamine, the substance responsible for the characteristic odor of bunt spores. If the difference between total base and ammonia be considered as a rough measure of the amount of trimethylamine

present in the spores, it would appear probable that the spores of *Tilletia tritici* contain very little, if any, of this substance. Judged by the chemical evidence presented in Table I, the only collection of *Tilletia tritici* spores which might be expected to contain trimethylamine is that grown on the variety Ceres. The distillate collected from 10 gm. of these spores was evaporated to dryness and extracted with chloroform. The chloroform extract was then dried, and a few drops of 0.1 N sodium hydroxide were added to it, but no odor of trimethylamine could be detected.

The *Tilletia tritici* spores collected on Little Club, Kota, and Reward wheat gave very small differences between the total volatile base and the ammonia, the values being of about the same order of magnitude as that obtained for *Ustilago tritici*, the loose smut of wheat, the spores of which lack any fishy odor. The experimental evidence available, therefore, seems to support the observation already mentioned, that the spores of *Tilletia levis* alone liberate the characteristic odor usually attributed to bunt spores.

It has been shown by several workers that *Tilletia tritici* comprises several physiologic forms which differ from one another in their parasitic capabilities. The possibility exists that certain of these forms may be able to produce trimethylamine, whereas others may not. The spores of *Tilletia tritici* used in this investigation were considered to belong to a single physiologic form. However, if the hypothesis just suggested is correct, other forms of this fungus may occur, the spores of which contain trimethylamine.

Isolation of Trimethylamine

The acidified *Tilletia levis* distillates not required for ammonia determinations were collected in a flask to which a few drops of toluene had been added. The total distillate, representing 130 gm. of spores, was evaporated under reduced pressure. The residue was taken up with a little water, made alkaline with 20 cc. of concentrated sodium hydroxide, and distilled into 10 cc. of N hydrochloric acid. This procedure removed the methyl red used in titrating the total volatile base. The distillate was then evaporated to dryness under reduced pressure. The residue was transferred with a little water to a small beaker, was evaporated on the steam bath, and finally dried in a vacuum desiccator over con-

centrated sulfuric acid. A few sticks of sodium hydroxide were placed in the desiccator to remove traces of hydrochloric acid present in the residue.

The dried residue was extracted with hot chloroform. This extract was filtered, evaporated, dried in a vacuum desiccator, and weighed. The dried material was dissolved again in hot chloroform, filtered, evaporated, and redissolved in absolute alcohol. A small excess over the calculated quantity of a concentrated chloroplatinic acid solution was added to the alcoholic solution. The yellow precipitate produced was filtered off, washed with absolute alcohol, and redissolved in water. The aqueous solution was filtered, evaporated, and cooled. The orange-colored crystals which separated were filtered off, and redissolved in water. Final crystallization was effected by the addition of absolute alcohol to this solution. The mother liquor was concentrated, and two further small crops of crystals were obtained.

Micro analyses[2] of the first crop of crystals gave the following results: C 13.93, 13.92; H 3.82, 4.15 per cent. These values are in reasonably close agreement with those required by theory for trimethylamine chloroplatinate, which are C 13.63, H 3.82 per cent.

The difference between the total volatile base and the ammonia present in the distillate collected from the 130 gm. of spores was equivalent to 9.4 mg. of nitrogen. If ammonia and trimethylamine were the only two substances present, this weight of nitrogen would correspond to 40 mg. of trimethylamine. It is probable, however, that a closer approximation to the trimethylamine content of the spores is given by the weight of the dried hydrochloride obtained from the first extraction with chloroform, which was 25.4 mg., corresponding to 15.7 mg. of trimethylamine. The weight of crystalline trimethylamine chloroplatinate obtained was 21.1 mg., which represents 4.7 mg. of trimethylamine. Consequently 100 gm. of *Tilletia levis* spores might be expected to contain between 3.6 and 12 mg. of trimethylamine.

It should be recalled that the spores of *Tilletia levis* used in these experiments had been kept in the laboratory for several months before being used. When freshly collected they had a distinct fishy odor but, by the time the experiments were begun, this

[2] Analysis by Research Service Laboratories, New York.

odor had practically disappeared. This change indicated that the spores might be sufficiently alkaline in reaction to liberate trimethylamine. The hydrogen ion concentration of the different lots of spores was therefore determined, and is given in Table II. The spores of *Tilletia levis*, with the exception of those grown on the variety Ceres, are slightly more alkaline than those of *Tilletia tritici*. All of the collections of spores, however, have reactions sufficiently near the neutral point to permit the gradual liberation of both trimethylamine and ammonia. Had freshly collected spores of *Tilletia levis* been available for the isolation of trimethylamine it is probable that a higher yield of this substance would have been obtained.

TABLE II

Reactions of Water Suspensions of Wheat Smut Spores as Determined by Quinhydrone Electrode

Wheat variety	Smut	Reaction
		pH
Little Club	*Tilletia levis*	6.23
" "	" *tritici*	6.11
Kota	" *levis*	6.20
"	" *tritici*	6.08
Reward	" *levis*	6.25
"	" *tritici*	6.15
Ceres	" *levis*	6.28
"	" *tritici*	6.28
Several	*Ustilago* "	5.96

SUMMARY

The amount of total volatile base and ammonia obtainable from a collection of *Tilletia levis* or *Tilletia tritici* spores depends upon the wheat variety on which the spores have developed. The ammonia content of the spores varied from 54 to 143 mg. per 100 gm. of spores.

Distillates from *Tilletia levis* spores have a higher content of total volatile base and show greater differences between total volatile base and ammonia than do those from *Tilletia tritici* spores.

Freshly collected spores of *Tilletia levis* emit an odor resembling that of herring brine. Trimethylamine was isolated from these spores, and it is considered to be the substance responsible for

their odor. The trimethylamine content of these spores was probably between 3.6 and 12 mg. per 100 gm. of spores.

Spores of *Tilletia tritici*, even when freshly collected, did not emit the odor of trimethylamine and none of this substance could be detected in distillates obtained from these spores.

THE DESTRUCTION OF CHOLESTEROL BY THE ANIMAL ORGANISM*

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Historically, at least, cholesterol has been considered a mother substance in sterol metabolism. It therefore seemed peculiarly important to us to determine whether cholesterol was broken down by the animal. In experiments designed to alter the cholesterol content of the brain and vascular system by the feeding of cholesterol, we were struck by the great variation in response of the different animals both as regards species and variations within the species. Since cholesterol is quite easily absorbable, it seemed curious that we were unable in some animals to produce any pronounced histological changes in the organs in spite of the feeding of very large amounts of that substance. It seemed possible that if all or most of the sterol was absorbed, the animals must either deposit the sterol or change it into some other form. We, therefore, have made balance experiments on rabbits to determine the truth or falsity of this supposition.

Method

Rabbits were kept in metabolism cages and fed with hay, oats, and beet-root. The cholesterol was administered by stomach tube as a 5 per cent solution dissolved in beet-root oil.

Determinations of cholesterol were made by means of the Windaus digitonin method (1). The concentration of sterol employed in the precipitation was somewhat smaller than that given in the original method. 5 to 10 mg. in 10 cc. of solution

could always be determined with an error of less than 3 per cent. Every analysis was carried out at least twice.

For saponification 3 to 4 per cent sodium ethylate was employed at water bath temperature for 3 hours. Repeated determinations show that the loss of sterol caused by the saponification was a maximum of 5 per cent.

The largest variations occurred in the hot saponification of the non-saponifiable fraction of feces prepared by preliminary cold saponification following the method of Kossel and Obermüller (2). The variations in this fraction may be due to esters which are difficult to saponify (see Page and Rudy (3)) or to the saponification which can render the sterol non-precipitable by digitonin.

We have always employed the 3 hour saponification; and since the average loss by the procedure is 5 per cent, we have added this correction to the analytical cholesterol values of both organ and feces.

Feces Analysis—Feces were collected weekly, carefully separated from the hay, and thoroughly ground. 10 gm. were dried overnight at 100°, boiled for 5 minutes with absolute alcohol, cooled, filtered, and aliquot portions saponified. The 10 gm. of feces had a specific gravity of about 1, so that in 200 cc. extraction flasks about 190 cc. of alcohol were employed. The extraction is probably practically complete because the values agree closely with those found for ether extraction. Recovery of added cholesterol was within 1 per cent of the theoretical value.

The small amount of hay which remained in the cages after removal of the feces was collected and analyzed.

Total sterol excretion	Sterol content in hay	Sterol in total excretion
mg.	*mg.*	*per cent*
1310	44.3	3.4
1090	24.4	2.2
1330	30.9	2.3
1590	33.2	2.1
1790	27.6	1.6
2200	34.4	1.6

A correction of 3 per cent was therefore added to correct for loss from the small amount of hay remaining in the cage. Analyses of the urine collected over week periods showed no appreciable amount of sterol; therefore, no correction was made.

Organ Analysis—At the end of the experiment the animal was killed and divided as follows: (a) brain, (b) liver + gallbladder and bile, (c) kidneys and adrenals, (d) gastrointestinal tract with contents, (e) fur and skin, (f) residue of muscle, bones, lungs, etc. The portions indicated by (a) to (e) were treated for 2 to 3 hours on the water bath with 2 N NaOH, an aliquot portion of the homogeneous solution was thoroughly extracted with ether, and finally saponified with sodium ethylate. The residue (f) was ground in a meat grinder, dried at 80°, and powdered in a mill. An aliquot portion was treated with 60. per cent NaOH for 2 to 3 hours on the boiling water bath and then extracted with ether.

TABLE I

Cholesterol Content of Normal Rabbits

	Rabbit 1			Rabbit 2			Rabbit 3		
	Organ weight	Cholesterol		Organ weight	Cholesterol		Organ weight	Cholesterol	
	gm.	*mg.*	*per cent*	*gm.*	*mg.*	*per cent*	*gm.*	*mg.*	*per cent*
Brain..............	9	184	2.05	8	171	2.13	8.1	158	1.59
Liver + gallbladder and bile......	110	194	0.175	102.5	224	0.218	90	153	0.170
Kidneys and adrenals.............	14.8	56	0.378	19.8	95	0.480	10.7	44.3	0.414
Gastrointestinal tract............	520	520	0.100	530	800	0.151	540	544	0.101
Fur and skin.......	260	520	0.200	430	653	0.152	265	530	0.200
Residue............	950	875	0.092	1653	1240	0.075	1053	858	0.082
Total cholesterol...	1864	2359	0.126	2743	3183	0.116	1967	2287	0.116

Average total content = 0.119 per cent.

The residue of bone was dissolved with 10 per cent HCl, extracted with ether, and the extracts combined. A small addition of alcohol was usually necessary for the extraction with ether to make the layers separate sharply.

To determine whether loss occurred from the aqueous saponification a portion of (f) was analyzed with and without addition of cholesterol.

It was found that the addition of 5 per cent for the saponification certainly adequately corrects for any loss during the organ analysis.

Results

The cholesterol content of normal rabbits is given in Table I. *Normal Content of Sterol of Rabbit Feces*—The normal content

TABLE II

Balance Experiments I and II

Experi-ment No.	Date	Weight	Choles-terol fed	Amount of feces	Sterol in feces	
	1930	*gm.*	*gm.*	*gm.*	*mg.*	*per cent*
I*	Aug. 28	3460				
	" 31		1.00	160	570	0.358
	Sept. 4	3270				
	" 7		2.50	460	1840	0.399
	" 11	3170				
	" 14		2.50	280	1310	0.468
	" 21		2.50	360	1090	0.302
	" 24	3300				
	" 28		2.50	290	1330	0.460
	Oct. 3	2900				
	" 5		2.50	255	1590	0.625
	" 12	2750	2.50	220	1430	0.647
	" 19		2.50	260	1790	0.687
	" 20	2700				
	" 26		2.50	340	2200	0.648
	Nov. 2	2890	3.60	280	1790	0.638
	" 9		3.60	280	1950	0.695
	" 10	2850				
	" 16		3.60	175	1120	0.638
	" 23	2650	3.60	150	1240	0.825
Total....................			35.40	3510	19.25 gm.	
			2.53†		+0.58 "	(3% correc-tion for hay)
			37.93		+0.96 "	(5% correc-tion for saponifica-tion)
					20.79 "	

* Content at beginning, 3460 × 0.119 per cent = 4.12 gm. cholesterol. Normal excretion in feces (0.205 per cent) in 3510 gm. = 7.20 gm. sterol. Total excretion in feces = 20.79 gm. Excess excretion = 13.59 gm.

† Phytosterol in oil.

TABLE II—*Concluded*

Experiment No.	Date	Weight	Cholesterol fed	Amount of feces	Sterol in feces	
	1930	*gm.*	*gm.*	*gm.*	*mg.*	*per cent*
II‡	Nov. 10	2550				
	" 23		3.6	160	1040	0.650
	" 25	2350				
	" 30		3.6	175	1505	0.860
	Dec. 3	2250				
	" 7		3.6	150	1530	1.020
	" 14	1850	3.6	120	1500	1.250
Total.....................			14.40	605	5.58 gm.	
			1.03†		+0.17 " (3% correction for hay)	
			15.43		+0.28 " (5% correction for saponification)	
					6.03 "	

‡ Content at beginning, 2550 × 0.119 per cent = 3.02 gm. cholesterol. Normal excretion in feces (0.207 per cent) in 605 gm. = 1.25 gm. sterol. Total excretion in feces = 6.03 gm. Excess excretion = 4.78 gm.

was determined in a period just before the balance experiment and was found to be 0.207 per cent (Rabbit 2), 0.194 per cent (Rabbit 3), 0.222 per cent (Rabbit 4), and 0.196 per cent (Rabbit 5).

For Experiment I the preliminary period was not carried out, hence the average of the above periods was taken for this one experiment. The phytosterol content of the beet oil is about 0.35 per cent. Our analysis showed an average of 357.8 mg. for 100 cc. of oil.

Balance Experiments

In Tables II and III are found the amount of cholesterol administered, that excreted, per cent in feces, and the weight of the animals.

Experiment III is not reported in detail. Suffice it to say that 18.0 gm. of cholesterol were fed, that the corrected excretion was

TABLE III

Balance Experiments IV and V

Experi- ment No.	Date	Weight	Choles- terol fed	Amount of feces	Sterol in feces	
	1931	*gm.*	*gm.*	*gm.*	*mg.*	*per cent*
IV*	Jan. 4	3150				
	" 18	3080	2.5	255	1340	0.525
	" 25	2965	2.6	175	1300	0.743
	Feb. 1	2840	2.5	190	1370	0.722
	" 8	2800	2.5	170	1590	0.935
	" 15	2630	2.5	100	1220	1.220
	" 22	2340	2.5	55	900	1.640
	" 25	2305				
	" 29	2445		165	1250	0.756
	Mar. 3	2230	2.5	32	165	0.516
Total....................			17.60 +1.26† 18.86	1142	9.14 gm. +0.27 " (3% correc- tion for hay) +0.46 " (5% correc- tion for saponifica- tion) ———— 9.87 "	
V‡	Jan. 11	3340				
	" 18	3180	2.5	340	1585	0.467
	" 25	3170	2.6	335	1960	0.585
	Feb. 1	2790	3.6	230	2160	0.942
	" 8	2690	3.5	245	1925	0.785
	" 15	2770	2.0	150	1195	0.798
	" 22	2840		330	1110	0.333
	" 25	2900				
	" 29	2835	2.5	235	1850	0.790
	Mar. 7	2740	2.5	250	2050	0.830
	" 11	2620				

* Content at beginning, 3150 × 0.119 per cent = 3.75 gm. cholesterol. Normal excretion in feces (0.222 per cent) in 1142 gm. = 2.54 gm. sterol. Total excretion in feces = 9.87 gm. Excess excretion = 7.33 gm.

† Phytosterol in oil.

‡ Content at beginning, 3340 × 0.119 per cent = 3.97 gm. cholesterol. Normal excretion in feces (0.196 per cent) in 2440 gm. = 4.40 gm. sterol. Total excretion in feces = 18.30 gm. Excess excretion = 13.90 gm.

TABLE III—*Concluded*

Experiment No.	Date	Weight	Cholesterol fed	Amount of feces	Sterol in feces	
	1931	*gm.*	*gm.*	*gm.*	*mg.*	*per cent*
V‡	Mar. 14	2510	2.5	195	1550	0.795
	" 21	2340	2.0	130	1605	1.233
Total:....................			22.70 +1.62† ——— 24.32	2440	16.94 gm. +0.51 " (3% correction for hay) +0.85 " (5% correction for saponification) ——— 18.30 "	

9.59 gm., normal fecal excretion was 1.72 gm., and the total fecal excretion was 6.03 gm. of sterol. Thus the excess excretion of sterol amounted to 7.87 gm.

The analysis of the whole animal at the end of the experiment is given in Table IV. At the end of Table IV will be seen the figures of the total corrected (for saponification) cholesterol content.

From the normal content and content of cholesterol in the animal at the end of the experiment can be calculated the deposition of sterol in the organism.

Experiment No.	Cholesterol content at beginning	Cholesterol content at end	Sterol deposition
	gm.	*gm.*	*gm.*
I	4.12	4.70	0.58
II	3.02	7.22	4.20
III	3.06	9.04	5.98
IV	3.75	6.95	3.20
V	3.97	7.02	3.05

The cholesterol balance is taken as the difference between the sterol fed on the one hand and the sterol excreted, plus that found in the body, on the other. The assumption is made that the normal excretion of sterol continues in spite of the fact that sterol is

TABLE IV

Organ Analysis for Cholesterol

Experiment No.		Brain	Liver + gallbladder and bile	Kidneys + adrenals	Gastrointestinal tract	Fur and skin	Residue	Total cholesterol
I	Organ weight, gm	9.6	110	19.2	620	325	1541	2625
	Cholesterol, mg	208	770	177.3	1430	800	1100	4480
	" per cent	2.160	0.700	0.922	0.231	0.246	0.071	0.170
II	Organ weight, gm	9.5	112	16.6	371	307	1090	1806
	Cholesterol, mg	187	995	107	2150	1220	2220	6880
	" per cent	1.970	0.890	0.645	0.580	0.398	0.224	0.380
III	Organ weight, gm	9.5	108	21.9	445	295	1000	1880
	Cholesterol, mg	176	1370	219	2230	1170	3320	8610
	" per cent	1.850	1.270	1.000	0.525	0.396	0.332	0.458
IV	Organ weight, gm	9.4	68.1	20.8	458	400	1078	2034
	Cholesterol, mg	171	328	221	2290	1275	2335	6620
	" per cent	1.81	0.482	1.103	0.500	0.319	0.216	0.324
V	Organ weight, gm	10.4	52.9	25.3	507	395	1280	2271
	Cholesterol, mg	213	709	303	1220	1230	3020	6690
	" per cent	2.04	1.33	1.195	0.240	0.311	0.237	0.294

Experiment No.	I	II	III	IV	V
Total sterol found, gm	4.48	6.88	8.61	6.62	6.69
5 per cent correction (saponification), gm	0.22	0.34	0.43	0.33	0.33
Corrected total content, gm	4.70	7.22	9.04	6.95	7.02

fed. This seems justified especially as Schönheimer (4) has shown that the sterol of normal rabbit feces is for the most part composed of phytosterol from the food. However, as a control, we have calculated the cholesterol degradation ignoring the normal excretion of sterol in the feces. These uncorrected figures are given in parentheses in Table V.

In Column 6 is given the difference between administered and found cholesterol.

DISCUSSION

Regardless of whether or not the normal excretion of sterol is considered in the balance experiment, it is clear from Table V that

TABLE V

Calculated Cholesterol Destruction

Experiment No. (1)	Total administered cholesterol (1)	Excess excretion (2)	Total excretion (3)	Deposition in animal (4)	Total cholesterol found (5)	Cholesterol destruction ((1) − (5)) (6)	Length of experiment (7)
	gm.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*wks.*
I	37.93	13.59	(20.79)	0.58	14.17 (21.37)	23.76 (16.56)	13
II	15.43	4.78	(6.03)	4.20	8.98 (10.23)	6.45 (5.20)	4
III	19.28	7.87	(9.59)	5.98	13.85 (15.57)	5.43 (3.71)	5
IV	18.86	7.33	(9.87)	3.20	10.52 (13.07)	8.33 (5.79)	7
V	24.32	13.90	(18.30)	3.05	16.95 (21.35)	7.37 (2.97)	9

The figures given in parentheses are uncorrected values calculated for cholesterol degradation, the normal excretion of sterol in the feces being disregarded.

large amounts of cholesterol disappear. Calculated as the amount destroyed per week the results are as follows: Experiment I 1.8 gm., Experiment II 1.6 gm., Experiment III 1.1 gm., Experiment IV 1.2 gm., and Experiment V 0.8 gm.

Because of the different amounts of sterol fed and variations in the length of the experiments, we are not able to determine whether the breakdown is a constant characteristic for cholesterol. What evidence we have indicates large individual and species variations in the ability of the animal to degrade cholesterol.

It is interesting to note that the deposition of cholesterol occurred in all organs of the body with the exception of the brain.

Even the content of sterol in the skin and fur rose 50 to 100 per cent.

By continuous feeding of sterol the content in feces rises gradually until it seems that an equilibrium is reached between the content of sterol stored in the animal and that excreted. In general the greater the content of sterol in the feces the richer the body is in that substance.

These experiments seem to us important for the problem of cholesterol atherosclerosis, both experimental and human. They demonstrate that an endogenous as well as an exogenous sterol metabolism must be reckoned with. It seems demonstrated that from feeding alone the sterol content of most organs other than the brain can be raised and that there appears to be an attempt at the establishment of an equilibrium either by virtue of an increased destruction of cholesterol or hindrance to further absorption.

We are now investigating the breakdown products found in the feces and blood following cholesterol feeding. It is of interest that from arteriosclerotic plaques from the aorta that Page and Menschick (5) have identified the ketone, cholestenone. Balance experiments are being continued on various animal species in the hope that it may offer some plausible explanation of why cholesterol sclerosis is so variant among the animal species.

SUMMARY

1. Complete balance experiments were carried out with rabbits fed on large amounts of cholesterol.

2. It could be shown that destruction of cholesterol of from 0.8 to 1.8 gm. per week occurred.

3. Administered cholesterol is deposited throughout the body except in the brain.

4. There appears to be an equilibrium established between the content of sterol in the organs, in the excreta, and probably in the food.

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A STUDY OF THE MILK, BLOOD, AND EXCRETA OF COWS FED MODERATE AND EXCESSIVE AMOUNTS OF IRRADIATED YEAST OR ERGOSTEROL

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It was recently shown that infantile rickets could be prevented or cured by means of cow's milk which has been rendered highly antirachitic by supplementing the ration with irradiated yeast or with irradiated ergosterol (viosterol) (1). Milk of this kind, when given to a large series of infants during the winter, prevented rickets or brought about calcification of the rachitic epiphyses within a month. From the point of view of the number of anti-rachitic rat units included in the ration, it was of interest that feeding irradiated yeast to the cow led to elaboration of a decidedly more potent milk than the viosterol. This distinction was evident by laboratory assay and clinical test. The superiority of this method of antirachitic therapy is that it functions automatically, as the specific factor is incorporated in the regular diet of the infant.

This study has been continued and extended in several directions. In the first place, excessive as well as moderate amounts of these two antirachitic agents have been fed. Furthermore, the milk has been analyzed not only for vitamin D, but also for calcium and inorganic phosphorus, and an investigation carried out to ascertain the relation of this vitamin to the total daily secretion and to the fat content of the milk of individual cows. Analyses of calcium and phosphorus were carried out on the blood, and the excretion of vitamin D was determined in the urine and in the feces. At the same time observations were made in regard to the health of the cows, especially of those which were receiving excessive amounts of the irradiated yeast and ergosterol, and, finally his-

tologic sections of various organs were prepared from animals which had received large amounts of the antirachitic agents for a period of almost a year. For cooperation in this work we wish to thank Dr. Logan T. Wilson of the Walker-Gordon Laboratories.

The milk which was used in the clinical tests and found most satisfactory was that procured from cows which received irradiated yeast having a potency of 15 to 25 D, which is equivalent to approximately 200 to 330 units per gm. On repeated assay
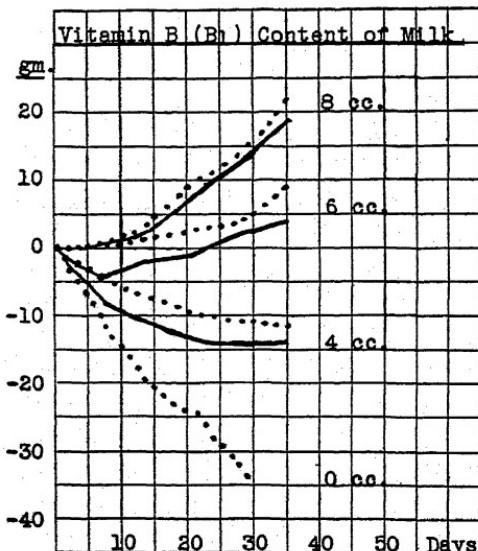
CHART 1. Growth of rats on a vitamin B-free ration, when varying amounts of milk were added. The dotted lines represent gains on supplements of ordinary milk; the continuous lines represent gains on supplements of vitamin D "yeast milk."

(butter fat being used for this purpose), it was found to contain 150 to 160 rat units per quart. About 280 gm. of yeast were fed to each cow daily, the yeast being incorporated in the grain concentrates, which amounted to 9 to 10 pounds. In view of the fact that this yeast was rich in vitamin B, containing 12 to 15 Sherman units per gm., so that each cow received about 3500 units daily, we had expected that the milk would be exceedingly rich in this vitamin. Repeated bioassays showed, however, that the titer of vitamin B was not increased in the milk by this procedure. This

phenomenon is well illustrated by Chart 1, which shows that vitamin B was not enriched in the milk, although it was contained in large amount in the yeast; each curve indicates the average gain of seven to nine animals. It would seem that the water-soluble vitamins, in contradistinction to the fat-soluble vitamin D, are not readily increased beyond the level found in this milk.

The effect of moderate supplements of irradiated ergosterol or of yeast on the calcium and phosphorus concentration of the milk and of the blood serum are reproduced in Table I. It seems unnecessary to discuss these results as they show merely that these supplements do not change the phosphorus, calcium, or ash content of the milk, or the phosphorus and calcium concentrations of the serum.

TABLE I

Moderate Supplements of Irradiated Ergosterol and Yeast

The results are expressed in mg. per 100 cc.

	Serum		Milk		
	Ca	P	Ash	Ca	P
Controls..........................	10.9	6.3	740	116	91
Irradiated yeast					
30,000 rat units..................	11.1	6.5	740	114	90
60,000 " "	10.6	7.0	730	114	91
Irradiated ergosterol					
100,000 rat units................	10.9	5.5	740	118	92
200,000 " "	10.4	5.8	750	127	95

As stated, a few cows were fed very large amounts of the yeast and of the ergosterol in order to note their reaction to doses which must be regarded as toxic rather than therapeutic. Three cows were given 4 pounds (1800 gm.) of irradiated yeast daily. This amount contained over 600,000 rat units. The regular therapeutic feedings of yeast were 8 to 10 ounces (280 gm.) daily per capita or 60,000 to 90,000 units. As the cows consumed on an average only three-fourths of the grain supplement in which the yeast was incorporated, they received 450,000 rather than 600,000 rat units daily. Similarly, in respect to the irradiated ergosterol which was dissolved in corn oil and added to the grain, although the cows were given 2,000,000 rat units per day, they took only

about 1,500,000 units.   The effect of these pathological amounts of vitamin D on the serum and milk of the cows may be seen in Table II.   The results, in brief, were that a slight but decided rise in the calcium of the serum came about from feeding each of the vitamin supplements, and that the increases of inorganic phosphate in the serum were much more marked.   This distinction was to be anticipated, in view of the fact that the ration of the cows was comparatively high in phosphorus and low in calcium.   As regards the milk, there was a slight increase both in total ash and in

TABLE II

Excessive Supplements of Irradiated Ergosterol and Yeast

The results for Ca, P, and ash are expressed in mg. per 100 cc.; for phosphatase, in units.

Breed	Supplement (vitamin D units)	Serum			Milk			Butter, gm. per vitamin D unit
		Ca	P	Phos-phatase	Ash	Ca	P	
Holstein	Irradiated	11.8	11.4	0.179	700	124.0	86.4	
Jersey	ergosterol	12.4	9.0		759	134.2	93.3	
"	1,500,000	11.7	7.8		776	115.6	102.8	
Guernsey		12.9	11.4	0.289	800	141.8	107.0	0.02
Holstein	Irradiated	12.1	9.4		690	116.0	82.8	
Jersey	yeast 450,000	11.0	8.7	0.064	744	130.5	107.5	
Guernsey		11.6	9.3	0.082	785	154.6	108.8	0.04
Holstein	Controls	10.0	5.2		682	107.9	68.5	
Jersey		10.8	5.5	0.095	700	119.8	84.8	
Guernsey		11.4	5.5	0.068	709	114.2	86.1	8.0

calcium and phosphorus.   In interpreting these data, more especially those relating to milk, the breed of the cows must be taken into consideration as this factor is of importance in relation to the daily output of milk.   For example, it may be noted that the figures for calcium and phosphorus are comparatively low for the milk of the Holstein cows, which is probably to be attributed to the fact that cows of this breed secrete a large quantity of milk. Therefore, cognizance must be taken not only of vitamin intake, but of breed.

In order to determine in what concentration vitamin D was

present in the blood of the cows, specimens were obtained from those receiving moderate and excessive amounts of irradiated yeast and an excessive amount of irradiated ergosterol. Furthermore, separate examinations were made of the plasma and of the corpuscles; as the latter were not washed, they contained a small percentage of plasma. The results of these bioassays may be seen in Table III. It will be noted that, as expected, the plasma contained considerably more vitamin than the corpuscles. In the

TABLE III

Antirachitic Activity of Blood of Cows Fed Irradiated Yeast or Ergosterol

Cow No.	Vitamin D		Gm. containing 1 unit vitamin		
	Units	Supplement	Plasma	Corpuscles	Blood
5585	1,500,000	Irradiated ergosterol	1	4	1.5
8189	450,000	" yeast	1	4	1.5
89	100,000	" "	2	5	2.5

TABLE IV

Vitamin D Excreted in 24 Hours in Milk, Feces, and Urine by Cow Receiving 300 Gm. (100,000 Units) of Irradiated Yeast Daily

Material assayed	Amount excreted in 24 hrs.	Amount containing 1 unit vitamin D	Total units excreted
	lbs.	*gm.*	
Milk..............................	40	8.0	2,362
Feces.............................	90.4	1.5	27,361
Urine.............................	19	50.0*	0

* No antirachitic activity found in this amount.

blood of the cows receiving excessive amounts of either ergosterol or yeast, 1 unit of vitamin D was contained in 1.5 gm. of whole blood, whereas in the blood of those receiving a moderate dosage of yeast, 2.5 gm. of whole blood contained but 1 unit. On calculation, it is found that feeding moderate amounts of irradiated yeast to a cow of average weight results in approximately 9000 units of vitamin D being carried in the blood of the animal.

For the tests of excreta only such cows were used as were being fed about 300 gm. of irradiated yeast; in other words, the amount given for commercial purposes. To this end the urine and feces for a 24 hour period were collected and aliquots taken for tests.

At the same time as the excreta were assayed, milk was taken for biological assay. A summary of this part of the investigation is reproduced in Table IV. As was expected, none of this vitamin was recovered from the urine. Assays of the feces showed, however, that approximately 25 per cent of the vitamin D ingested daily was eliminated by the bowel. Whether this amount is unabsorbed through a failure of the body to digest the yeast or whether the blood is unable to hold such large amounts in solution and excretes the excess through the bowel, cannot be judged. It may be added that Klein and Russell (2) found that chickens given cod liver oil or irradiated ergosterol, excreted a surprisingly large proportion of the intake in the feces.

An opportunity was presented of assaying the liver of one of the cows which had been fed excessive amounts of irradiated yeast for a long period. The organ was obtained immediately after slaughtering the animal and tested by the usual method. The fresh liver tissue was found to contain more than 1 unit of vitamin D per gm. If the figures of Sherman, namely that beef livers contain from 3 to 5 per cent of fat, are taken as a basis for calculation, the oil from this liver must have contained from 20 to 30 units of vitamin D per gm. This degree of activity is greater than that of average cod liver oil.

As far as noted, excessive feeding of irradiated yeast or of irradiated ergosterol did not have a harmful effect on the health of the cows; their appearance, weight, and appetite remained unchanged. In several cases various organs were subjected to histological examination, in order to note particularly whether evidences of calcification could be found. For this purpose sections from the aorta, kidneys, and liver were examined under the microscope. The result of this study may be summarized by the statement that no signs of calcification were observed. In two instances, one a control animal, thickening of the intima of the aorta was noted, with some increase in fat and in calcium. The fifth and eleventh ribs of two animals were ashed to ascertain whether a decrease in ash had resulted from excessive ingestion of vitamin D. An ash content varying from 62.7 to 65.4 per cent was found and was interpreted as normal. However, these figures do not preclude the possibility that some calcium and phosphate had been withdrawn from the skeleton.

In view of the fact that "vitamin D milk" is being adopted to an increasing degree, it seemed of value to ascertain to what extent the total milk production of the individual cow and the percentage of butter fat in the milk affects the concentration of the vitamin in the milk. It is evident that knowledge of these relationships might prove of importance in evolving an economical and uniform method of production. To obtain information on these points, milk was obtained from cows which were receiving the same amounts of vitamin D in the form of irradiated yeast. Cows of three different kinds were selected for comparison; one which gave great production of milk containing low butter fat, a second group which gave low production of milk with low butter fat, and a third which gave low production with high butter fat. The

TABLE V

Effect of High and Low Production and of High and Low Butter Fat on Vitamin D Content of Milk. Cows Fed 300 Gm. of Irradiated Yeast Daily

Cow No.	Milk	Butter fat			Units vitamin D in milk	
					Per qt.	Per day
	lbs. per day	*per cent*	*lbs. per day*	*units per gm.*		
A1775	26.7	3.6	0.96	4.5	158	1896
5666	39.4	3.3	1.3	4.0	128.8	2293
7147	26.7	4.7	1.2	3.6	165	1980
Control	30.0	4.0	1.2	0.125	5	70

butter fats from these various milks were assayed for their antirachitic activity and the units of vitamin D per quart calculated. The results, which seem both interesting and significant, are reproduced in Table V. In the first place, they showed, within the limits of this experiment, that the lower the total production of butter fat the higher will be the concentration of vitamin D in the fat, and, furthermore, that the greater the daily production the greater will be the total number of vitamins D units excreted in the milk per day, although the units per quart may be lower.

When we consider these results from the point of view of economical production of "vitamin D milk," it is evident that the most advantageous method is to feed the herd irradiated yeast on what may be termed a percentage production basis; in other words,

not to feed each cow a fixed amount of vitamin D supplement daily, irrespective of individual production but to feed according to the daily output of milk of each cow. We had the opportunity of putting this conclusion to a practical test with some commercial herds. Table VI illustrates an experiment of this kind, showing the average milk production, the level at which the vitamin D supplement was fed, the number of units per quart, and the average total number of units per day excreted into the milk. It will be noted that when the percentage production method was followed, over 2500 units per day were obtained in a herd which gave the very high average daily production of 43.5 pounds per cow, whereas by the method of feeding each cow 300 gm. of yeast

TABLE VI

Relation of Total Milk Production of Herd to Total Vitamin D Content of Milk

Herd No.	Average milk production	Average butter fat	Amount of yeast fed	Units vitamin D in milk	
				Per qt.	Per day
	lbs.	*per cent*	*gm.*		
I	43.5	3.6	280	131	2685
II	31.8	4.2	240	135	1952
III	30.5	4.6	300	173	2560
IV	30.9	5.0	350	200	3000
V	27.3	4.0	300	168	2134
VI	45.2	3.9	350	160	3410

daily, a total of only 1844 to 2125 units was obtained. Furthermore, it is seen that this high total of units was brought about in milk which contained only 131 units of vitamin D per quart. Evidently, in establishing the level at which the vitamin D supplement should be fed, it is of decided advantage to be guided by the level of milk production of the herd.

For the bioassays of milk we have fed the rats either rendered butter fat or a concentrated cream, the percentage of butter fat of which has been determined. In assaying by this procedure, it is of importance to determine also the percentage of butter fat in the whole milk, inasmuch as the number of units of vitamin D per quart is dependent not only on the number of units per gm. of butter fat, but also on the number of gm. of butter fat per quart of milk.

SUMMARY

The milk of cows which received about 300 gm. (60,000 units) of irradiated yeast daily is highly antirachitic and prevents or cures rickets in infants. In spite of the very high concentration of vitamin B_1 in the yeast, the titer of this vitamin in the milk was not increased. No increase in the phosphorus, calcium, or ash was found, nor was there an increase in the inorganic phosphorus or calcium content of the serum.

When excessive, non-therapeutic, amounts of irradiated ergosterol were fed, a slight but definite rise in the calcium, phosphorus, and ash content of the milk resulted; these increases were less in the milk of cows which secreted a large volume of milk daily. There was also a rise in the concentration of calcium in the serum and, especially, of phosphorus.

When excessive quantities of irradiated yeast were fed, the blood contained approximately 1 unit of vitamin D per 1.5 gm.; the same amount was found in the blood of cows which had received about 3 times as much irradiated ergosterol.

Assays of the excreta of the cows receiving 300 gm. of irradiated yeast daily showed that about 25 per cent of the vitamin D ingested was eliminated by way of the intestine. No vitamin D was recovered from the urine.

Histological examination of various organs of the cows which had received excessive amounts of irradiated ergosterol for long periods failed to show any lesions of the cellular structure or of the blood vessels. Analyses of the ash of the bones showed the percentages to be within normal limits.

Investigation as to what extent the total milk production of the cow and the percentage of butter fat in the milk affect the concentration of vitamin D in the milk, showed that the lower the total production of butter fat the higher will be the concentration of the vitamin in the fat and, furthermore, that the greater the daily production the greater will be the total number of vitamin D units excreted in the milk per day, although the units per quart may be lower.

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2. Klein, D., and Russell, W. C., J. Biol. Chem., **93**, 693 (1931).

CONFIGURATIONAL RELATIONSHIPS OF METHYL-PHENYL-, METHYLCYCLOHEXYL-, AND METHYL-HEXYLCARBINOLS AND OF THEIR HOMOLOGUES

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From observations made in this laboratory in the course of several years, it became evident that if homologous series with respect to R_1 or R_2 are derived from substances of the type

$$
\begin{array}{c}
R_1 \\
| \\
H\!-\!C\!-\!X \\
| \\
R_2
\end{array}
$$
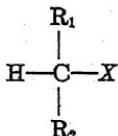
where X is either an alkyl group or another functional group, or a group possessing an absorption band in the ultra-violet region nearest to the visible, then the increment in the molecular rotation of each consecutive member is in a direction characteristic for the entire series; that is, if the rotation of the second member shows an increment in rotation towards the right, then the rotation of the third member likewise has an increment towards the right, etc., regardless of whether the individual members are dextrorotatory or levorotatory. On the basis of this rule, it is now possible to arrange substances in configurationally related homologous series, in such cases where the arrangement by direct chemical methods offers great technical difficulties. However, occasionally, un-certainties may arise as in the case given in Column 4 of Table I. For the first member of the methylisopropylcarbinol, the dextrorotatory member is chosen, even though the levorota-tory member might have equally well answered the requirement, inasmuch as the increment of the value of the rotation of the

TABLE I

Maximum Molecular Rotations of Configurationally Related Aliphatic Carbinols (in Homogeneous State). $[M]_D^{25}$

Methyl series (1) Carbinol	Phthalate (in alcohol)*	Acetate	Ethyl series (2) Carbinol	Phthalate (in alcohol)†	n-Propyl series (3) Carbinol	Phthalate (in alcohol)	Isopropyl series (4) Carbinol	Phthalate (in alcohol)‡	Isobutyl series (5) Carbinol	Phthalate (in alcohol)
CH_3 / HC—OH / CH_3 = 0			CH_3 / HC—OH / C_2H_5 = +10.3	+86.5	CH_3 / HC—OH / $C_3H_7\,(n)$ = +12.1	+90.0	CH_3 / HC—OH / $C_3H_7\,(iso)$ = +4.7	+230	CH_3 / HC—OH / $C_4H_9\,(iso)$ = +21.1 (?)	+134.5
CH_3 / HC—OH / C_2H_5 = −10.3	−86.5	−29.7	C_2H_5 / HC—OH / C_2H_5 = 0	0	C_2H_5 / HC—OH / $C_3H_7\,(n)$ = +5.1	+18.7	C_2H_5 / HC—OH / $C_3H_7\,(iso)$ = −16.7	+25	C_2H_5 / HC—OH / $C_4H_9\,(iso)$ = +24.6	+65.5
CH_3 / HC—OH / $C_3H_7\,(n)$ = −12.1	−90.0	−22.3	$C_3H_7\,(n)$ / HC—OH / C_2H_5 = −5.1	−18.7	$C_3H_7\,(n)$ / HC—OH / $C_3H_7\,(n)$ = 0	0	$C_3H_7\,(n)$ / HC—OH / $C_3H_7\,(iso)$ = −27.1	−58	$C_3H_7\,(n)$ / HC—OH / $C_4H_9\,(iso)$ = +16.3	+36.1
CH_3 / HC—OH / $C_4H_9\,(n)$ = −11.8	−117.5	−14.5	$C_4H_9\,(n)$ / HC—OH / C_2H_5 = −9.4	−51.5	$C_4H_9\,(n)$ / HC—OH / $C_3H_7\,(n)$ = −0.95	−6.7	$C_4H_9\,(n)$ / HC—OH / $C_3H_7\,(iso)$ = −35.9	−99	$C_4H_9\,(n)$ / HC—OH / $C_4H_9\,(iso)$ = +13.7	+23.6
$C_5H_{11}\,(n)$ / HC—OH / CH_3 = −12.0	−128.4	−13.0	$C_5H_{11}\,(n)$ / HC—OH / $C_2H_5\,(n)$ = −10.7	−60.2	$C_5H_{11}\,(n)$ / HC—OH / $C_3H_7\,(n)$ Levo	Levo	$C_5H_{11}\,(n)$ / HC—OH / $C_3H_7\,(iso)$ = −38.2	−129	$C_5H_{11}\,(n)$ / HC—OH / $C_4H_9\,(iso)$ = +11.3	

* Pickard, R. H., and Kenyon, J., J. Chem. Soc., 105, 848 (1914).
† Pickard, R. H., and Kenyon, J., J. Chem. Soc., 103, 1957 (1913).
‡ Kenyon, J., J. Chem. Soc., 105, 2233 (1914).
§ Levene, P. A., and Marker, R. E., J. Biol. Chem., 91, 418 (1931).

TABLE II

Maximum Molecular Rotations of Phenyl and Cyclohexylcarbinols and Their Derivatives. $[M]_D^{25}$

Phenyl series (1)			Cyclohexyl series (2)			Hexyl series (3)	
Carbinol	Phthalate (in alcohol)	Acetate	Carbinol	Phthalate (in alcohol)	Acetate	Carbinol	Phthalate (in alcohol)
CH_3—HC—OH—C_6H_5 −52.5*	+32.0	−194	CH_3—HC—OH—C_6H_{11} +6.5	+133.6	−4.9	CH_3—HC—OH—C_6H_{13} +12.7	+134.3
C_2H_5—HC—OH—C_6H_5 −39.4*	−10.6	−186	C_2H_5—HC—OH—C_6H_{11} −11.5	+38.4	−37.9	C_2H_5—HC—OH—C_6H_{13} +11.6	+28.0
C_3H_7 (n)—HC—OH—C_6H_5 −34.9	−21.0	−178	C_3H_7 (n)—HC—OH—C_6H_{11} −17.0	−4.8		C_3H_6 (n)—HC—OH—C_6H_{13} Dextro, lower than preceding member	Dextro
C_4H_9 (n)—HC—OH—C_6H_5 −28.2	−36.1	−163	C_4H_9 (n)—HC—OH—C_6H_{11} −21.9	−6.5			

* Pickard, R. H., and Kenyon, J., J. Chem. Soc., **99**, 45 (1911).

second member would have had a negative sign. However, in the second case the phthalates, which naturally should also constitute a homologous series, would not possess the requirements of such a series inasmuch as the increment of the second member would be to the right and that of the subsequent members to the left. Freudenberg[1] called attention to this circumstance and introduced this correction in our formulation of the rotations of the homologous members of the isopropylcarbinols. On the other hand, if the phthalates alone had been chosen for establishing the homologous series, uncertainty would have arisen regarding the second member inasmuch as either $-25°$ or $+25°$ would have satisfied the requirement of a series the members of which progressively increase to the left. Thus, a homologous series of carbinols is established more correctly on the basis of the maximum rotations of the individual carbinols and of the maximum rotations of the esters derived from the carbinols. On the grounds of these considerations, we are inclined to believe that the first member of the isobutyl series has not been resolved to the maximum.

Taking advantage of this rule, it was now found possible to correlate the secondary cyclohexylcarbinols with those of the corresponding hexyl carbinols. In Table II, Column 2, are given the rotations of the cyclohexylcarbinols, of the corresponding phthalates and acetates. By use of the same arguments as were applied in the case of the isopropyl series, the conclusion is reached that *the contribution of the cyclohexyl group has an opposite direction of rotation from that of the hexyl group when the members of the two series are configurationally related.* Again, as in the case of the isopropyl series, for the first member must be chosen the one with a dextrorotation, inasmuch as otherwise the phthalate of the first member may not satisfy the requirements of the first member of a homologous series.

The knowledge of the rotations of the members of the homologous series of secondary cyclohexylcarbinols necessitates a correction in regard to the views expressed by Levene and Stevens[2] regarding the correlation of methylcyclohexyl- and methylphenylcarbinols. Levene and Stevens assumed that all the members of

[1] Freudenberg, K., *Sitzungsber. Heidelberg. Akad. Wissensch.*, pt. 9 (1931).

[2] Levene, P. A., and Stevens, P. G., *J. Biol. Chem.*, **89**, 471 (1930).

the homologous series of cyclohexylcarbinols, the first member being methylcyclohexylcarbinol, rotated in the same direction. This assumption naturally led to the conclusion that methyl-phenylcarbinol rotated in the opposite direction from the other members of the homologous series. Inasmuch as the rotations of the members of the homologous series of cyclohexylcarbinols are as given in Table II, it follows that the members of the corresponding series of secondary phenyl carbinols all rotate in the same direction, as shown in Table II.

There are several other points of interest to be found on comparison of Columns 1 and 2 of Table II. It is seen that the rotations of the consecutive members of the phenyl series progressively increase towards the right (decrease in levorotation), whereas in the cyclohexyl series the increase is towards the left. The reason for the difference is due, with a great degree of probability, to the differences in the position and character of the radicles that determine the direction of rotation in the visible spectrum in the members of the two respective series. In the case of the phenyl series, it may be assumed that the phenyl group determines the direction in the visible spectrum and that the contribution of this group is levorotatory. The changes in the rotations of the consecutive members may be attributed to the increased dextrorotatory effect of the increasing weight of the aliphatic radicles. In the case of the members of the cyclohexyl series, on the other hand, the direction of rotation in the visible spectrum is probably determined by the contribution of the hydroxyl group. The increased levorotation of the consecutive members of this series is due to increasing weight of the non-cyclic radicle. Thus, in the two configurationally related series, the aliphatic groups function differently with regard to their effect on the rotation in the visible spectrum.

Passing now to phthalates, we observe that in both series the rotation in the visible spectrum is determined by the contribution of the phthalic residue and that the identical aliphatic groups produce a similar effect in the members of both series. Hence, the changes in rotation of the corresponding consecutive members are in the same direction. The similarity of the behavior of the homologous series of the phthalates given in Tables I and II may serve to indicate the way in which a comparison of shifts in the rotations of consecutive members of two homologous series of carbinols may

serve to establish their configurational relationship. The acetates, as seen from Tables I and II, may serve a similar purpose.

The observations reported in Table I, Column 4, are significant also in connection with the *Verchiebungssatz* of Freudenberg. According to this rule, two substances showing a shift of rotation in the same direction caused by an identical substitution are configurationally related. Table I shows that in many instances the rule actually holds, but it also shows that the rule is not without exception. Thus, in the isopropyl series, the first two members on substitution show a shift towards the right and the subsequent members a shift towards the left. Again, on comparison of the two configurationally related isopropyl and isobutyl series, it is observed that in the case of the first two members, the shift of the rotation on substitution is to the right, whereas in the subsequent members it is to the left in the case of the isopropyl series and to the right in the case of the isobutyl series. Thus it is evident that the *Verchiebungssatz* cannot be used as a general basis for the correlation of configurations.

EXPERIMENTAL

Dextro-Methylcyclohexylcarbinol—The inactive alcohol was prepared from cyclohexylmagnesium bromide and acetaldehyde.

The half phthalic ester of the inactive carbinol was resolved by crystallizing its strychnine salt from a large quantity of acetone. The maximum rotation of the ester obtained was

$$[\alpha]_D^{28} = \frac{+7.79° \times 100}{1 \times 16.008} = +48.6° \text{ (in absolute alcohol)}$$

The strychnine salt was decomposed with concentrated hydrochloric acid and the carbinol obtained by saponification of the phthalic ester. B. p. 105° at 35 mm. $D \frac{20}{4} = 0.919$.

$$[\alpha]_D^{28} = \frac{+4.70°}{1 \times 0.919} = +5.11°; [M]_D^{28} = +6.54° \text{ (homogeneous)}$$

4.558 mg. substance: 12.536 mg. CO_2 and 5.130 mg. H_2O

$$\begin{array}{lll} C_8H_{16}O. & \text{Calculated.} & C \; 74.9, \; H \; 12.6 \\ 128.13 & \text{Found.} & \text{"} \; 75.0, \text{"} \; 12.7 \end{array}$$

Levo-n-Propylcyclohexylcarbinol—The inactive carbinol was prepared from cyclohexylmagnesium bromide and *n*-butyric aldehyde.

The half phthalic ester of the inactive carbinol was resolved by crystallizing its strychnine salt from acetone cooled to 0°. Care should be taken when dissolving the salt, as a considerable amount of it is hydrolyzed if heated too long in acetone solution. The maximum rotation of the ester was

$$[\alpha]_D^{30} = \frac{-0.34°}{1 \times 21.88} = -1.55° \text{ (in absolute alcohol)}$$

The strychnine salt was decomposed and the carbinol obtained as described for methylcyclohexylcarbinol. B. p. 127° at 25 mm. $D \frac{29}{4} = 0.900$.

$$[\alpha]_D^{29} = \frac{-9.83°}{1 \times 0.900} = -10.92°; [M]_D^{29} = -17.03° \text{ (homogeneous)}$$

3.621 mg. substance: 10.162 mg. CO_2 and 4.222 mg. H_2O
$C_{10}H_{20}O$. Calculated. C 76.8, H 12.9
156.16 Found. " 76.5, " 13.0

Levo-Ethylcyclohexylcarbinol—The inactive carbinol was prepared from cyclohexylmagnesium bromide and propionic aldehyde.

The half phthalic ester of the inactive carbinol was resolved by crystallizing its strychnine salt from acetone. The resolution proceeded well when the alkaloid salt was allowed to crystallize from the acetone at room temperature. The maximum rotation of the ester was

$$[\alpha]_D^{29} = \frac{+3.75° \times 100}{1 \times 28.20} = +13.3° \text{ (in absolute alcohol)}$$

The strychnine salt was decomposed and the carbinol obtained as described for methylcyclohexylcarbinol. B. p. 106° at 19 mm. $D \frac{30}{4} = 0.911$.

$$[\alpha]_D^{30} = \frac{-7.37°}{1 \times 0.911} = -8.09°; [M]_D^{30} = -11.49° \text{ (homogeneous)}$$

5.051 mg. substance: 14.028 mg. CO_2 and 5.726 mg. H_2O
$C_9H_{18}O$. Calculated. C 76.0, H 12.7
142.13 Found. " 75.7, " 12.7

Levo-n-Butylcyclohexylcarbinol—The inactive carbinol was prepared from cyclohexylmagnesium bromide and *n*-valeric aldehyde.

The half phthalic ester of the inactive carbinol was resolved by crystallizing its strychnine salt from acetone cooled to −15°. This carbinol was much harder to resolve than the levo-*n*-propylcyclohexylcarbinol and the same precautions are necessary to prevent the hydrolysis of the salt. The maximum rotation of the ester was

$$[\alpha]_D^{20} = \frac{-0.22° \times 100}{1 \times 10.65} = -2.06° \text{ (in absolute alcohol)}$$

The strychnine salt was decomposed and the carbinol obtained as described for methylcyclohexylcarbinol. B. p. 135° at 25 mm. D $\frac{30}{4}$ = 0.893.

$$[\alpha]_D^{20} = \frac{-11.50°}{1 \times .893} = -12.87°; [M]_D^{20} = -21.88° \text{ (homogeneous)}$$

4.770 mg. substance: 13.591 mg. CO_2 and 5.620 mg. H_2O
$C_{11}H_{22}O$.   Calculated.   C 77.6, H 13.0
170.18   Found.   " 77.7, " 13.2

Levo-Propylphenylcarbinol (Maximum)—The acid phthalic ester of the inactive carbinol was dissolved in boiling methyl alcohol and an equivalent weight of strychnine was added. It was allowed to crystallize in a refrigerator. After eight crystallizations, the rotation of the free phthalic ester reached a constant value, $[\alpha]_D^{25} = -7.0°$ (in ether). The strychnine salt was decomposed with ammonia and the phthalate hydrolyzed with 1½ times the theory of 10 per cent potassium hydroxide solution. The free carbinol had the following rotation. B. p. 120° at 15 mm. D $\frac{24}{4}$ = 0.980.

$$[\alpha]_D^{24} = \frac{-21.8°}{1 \times 0.980} = -23.3°; [M]_D^{24} = -34.9° \text{ (homogeneous)}$$

$$[\alpha]_D = \frac{-6.80° \times 100}{1 \times 20 \times 0.95} = -35.8°; [M]_D^{24} = -53.6° \text{ (benzene)}$$

2.935 mg. substance: 8.594 mg. CO_2 and 2.450 mg. H_2O
$C_{10}H_{14}O$.   Calculated.   C 79.99, H 9.38
150.11   Found.   " 79.99, " 9.34

Dextro-n-Butylphenylcarbinol *(Maximum)*—The cinchonidine salt of the phthalic ester was crystallized from acetone until the free ester did not increase further in rotation. This required ten crystallizations and gave a phthalate of

$$[\alpha]_D^{25} = \frac{+2.60° \times 100}{1 \times 22.62} = +11.5° \text{ (ether)}$$

The carbinol was obtained in the usual way and was distilled. B. p. 130° at 15 mm. $D \frac{24}{4} = 0.966$.

$$[\alpha]_D^{24} = \frac{+16.65°}{1 \times 0.966} = +17.2°; [M]_D^{24} = +28.2° \text{ (homogeneous)}$$

$$[\alpha]_D^{24} = \frac{+4.15° \times 100}{1 \times 20 \times 0.663} = +31.3°; [M]_D^{24} = +51.3° \text{ (benzene)}$$

4.931 mg. substance: 14.590 mg. CO_2 and 4.250 mg. H_2O
$C_{11}H_{16}O$. Calculated. C 80.4, H 9.8
164.13 Found. " 80.7, " 9.7

Levo-Methylphenylchloromethane—65 gm. of methylphenylcarbinol, $[M]_D^{24} = -15.8°$ (homogeneous), were cooled in ice and 100 gm. of thionyl chloride were added. The product was heated 15 minutes on a steam bath, then fractionated. B. p. 90° at 26 mm. Yield 72 gm.

$$[\alpha]_D^{24} = \frac{-17.90°}{1 \times 1.059} = -16.9°; [M]_D^{24} = -23.7° \text{ (homogeneous)}$$

These values compare with those reported by Pickard and Kenyon for the maximum rotation of the chloride prepared from the carbinol of maximum rotation.

Dextro-3-Phenylbutyric Acid-4—7 gm. of sodium were dissolved in 100 cc. of absolute alcohol and 48 gm. of ethyl malonate added. To this were added 35 gm. of methylphenylchloromethane, $[M]_D^{24} = -8.72°$ (homogeneous). The product was refluxed for 3 hours, the malonate then hydrolyzed with potassium hydroxide, acidified with hydrochloric acid, and the malonic acid extracted with ether. The ether was distilled and the residue heated in a metal bath until CO_2 ceased coming off. The acid was then distilled, converted into the sodium salt, and extracted with ether. The

aqueous solution was acidified and the acid distilled. B. p. 134°
at 4 mm. Yield 26 gm. D $\frac{26}{4}$ = 1.066.

$$[\alpha]_D^{26} = \frac{+3.05°}{1 \times 1.066} = +2.85°; [M]_D^{26} = +4.67° \text{ (homogeneous)}$$

4.766 mg. substance: 12.890 mg. CO_2 and 3.030 mg. H_2O
$C_{10}H_{12}O_2$. Calculated. C 73.2, H 7.4
164.1   Found.   " 73.7, " 7.1

Dextro-Ethylphenylchloromethane—60 gm. of ethylphenylcar-
binol, $[M]_D^{24}$ = +24.67° (homogeneous), were cooled in ice and
50 gm. of thionyl chloride were added. The product was heated
on a steam bath for 15 minutes, and the excess thionyl chloride
was distilled off. Then the halide was distilled. B. p. 86° at
14 mm. Yield 31 gm. D $\frac{24}{4}$ = 1.039.

$$[\alpha]_D^{24} = \frac{+20.60°}{1 \times 1.039} = +19.82°; [M]_D^{24} = +30.62° \text{ (homogeneous)}$$

Dextro-3-Phenylvaleric Acid-5—23 gm. of sodium were dis-
solved in 250 cc. of absolute alcohol and 160 gm. of ethyl malonate
were added. To this were added 125 gm. of ethylphenylchloro-
methane, $[M]_D^{25}$ = −20.02°. The product was refluxed 2 hours,
then the ester was isolated, and the acid obtained as described for
2-phenylbutyric acid-4. B. p. 150° at 6 mm. Solid. Yield 95
gm.

$$[\alpha]_D^{24} = \frac{+1.32° \times 100}{1 \times 1.212 \times 20} = +5.44°; [M]_D^{24} = +9.70° \text{ (benzene)}$$

5.100 mg. substance: 13.962 mg. CO_2 and 3.735 mg. H_2O
$C_{11}H_{14}O_2$. Calculated. C 74.1, H 7.9
164.11   Found.   " 74.6, " 8.2

Levo-n-Propylphenylchloromethane—75 gm. of propylphenyl-
carbinol, $[M]_D^{24}$ = −18.71° (homogeneous), were cooled in ice and
100 gm. of thionyl chloride were added. The mixture was heated
15 minutes on a steam bath, then fractionated. B. p. 115° at 30
mm. Yield 82 gm. D $\frac{24}{4}$ = 1.004.

$$[\alpha]_D^{24} = \frac{-26.30°}{1 \times 1.004} = -26.19°; [M]_D^{24} = -44.15° \text{ (homogeneous)}$$

5.350 mg. substance: 14.090 mg. CO_2 and 3.830 mg. H_2O
$$C_{10}H_{13}Cl. \quad \text{Calculated.} \quad \text{C 71.2, H 7.8}$$
$$168.5 \qquad \text{Found.} \qquad \text{`` 71.8, `` 8.0}$$

Dextro-4-Phenylcaproic Acid-6—14 gm. of sodium were dissolved in 175 cc. of absolute alcohol and 95 gm. of ethyl malonate were added. To this were added 80 gm. of propylphenylchloromethane, $[M]_D^{24} = -18.71°$ (homogeneous). The acid was prepared and isolated as described for 3-phenylvaleric acid-5. B. p. 155° at 4 mm. Yield 49 gm. $D \frac{25}{4} = 1.025$.

$$[\alpha]_D^{25} = \frac{+8.15°}{1 \times 1.025} = +7.95°; [M]_D^{25} = +15.27° \text{ (homogeneous)}$$

3.877 mg. substance: 10.650 mg. CO_2 and 2.895 mg. H_2O
$$C_{12}H_{16}O_2. \quad \text{Calculated.} \quad \text{C 74.9, H 8.3}$$
$$192.13 \qquad \text{Found.} \qquad \text{`` 74.9, `` 8.3}$$

Levo-Butylphenylchloromethane—25 gm. of *n*-butylphenylcarbinol, $[M]_D^{25} = -13.14°$ (homogeneous), were cooled in ice and 50 gm. of thionyl chloride added. The product was heated on a steam bath for 15 minutes and then distilled. B. p. 120° at 15 mm. Yield 26 gm. $D \frac{25}{4} = 0.996$.

$$[\alpha]_D^{25} = \frac{-24.52°}{1 \times 0.996} = -24.62°; [M]_D^{25} = -45.06° \text{ (homogeneous)}$$

3.991 mg. substance: 10.454 mg. CO_2 and 2.810 mg. H_2O
$$C_{11}H_{15}Cl. \quad \text{Calculated.} \quad \text{C 72.3, H 8.3}$$
$$182.62 \qquad \text{Found.} \qquad \text{`` 71.5, `` 7.9}$$

Dextro-Methylphenylcarbinol Acetate—To 10 gm. of methylphenylcarbinol, $[\alpha]_D^{24} = +11.9°$ (homogeneous), were added 20 gm. of acetic anhydride, and the product was heated to 100° for 20 minutes. It was then fractionally distilled. B. p. 120° at 35 mm. Yield 8 gm.

$$[\alpha]_D^{24} = \frac{+4.05° \times 100}{1 \times 20 \times 0.70} = +28.9° \text{ (benzene)}$$

$$[\alpha]_D^{24} = \frac{+27.5°}{1 \times 1.025} = +26.8°; [M]_D^{24} = +43.95° \text{ (homogeneous)}$$

Calculated maximum: $[M]_D^{24} = -194°$ (homogeneous).

     4.016 mg. substance: 10.860 mg. CO_2 and 2.555 mg. H_2O
                 $C_{10}H_{12}O_2$. Calculated.   C 73.1, H 7.3
               164.1   Found.   " 73.7, " 7.1

Dextro-Ethylphenylcarbinol Acetate—To 20 gm. of ethyl-phenylcarbinol, $[M]_D^{21} = +21.2°$ (homogeneous), were added 40 gm. of acetic anhydride. The acetate was prepared as described for methylphenylcarbinol acetate. B. p. 130 at 35 mm. Yield 23 gm. $D \frac{24}{4} = 1.006$.

$$[\alpha]_D^{24} = \frac{+4.20° \times 100}{1 \times 20 \times 0.39} = +53.7° \text{ (benzene)}$$

$$[\alpha]_D^{24} = \frac{+55.67°}{1 \times 1.006} = +55.34°; [M]_D^{24} = +98.56° \text{ (homogeneous)}$$

Calculated maximum: $[M]_D^{24} = +186°$ (homogeneous).

     4.673 mg. substance: 12.830 mg. CO_2 and 3.340 mg. H_2O
                 $C_{11}H_{14}O_2$. Calculated.   C 74.1, H 7.9
              178.11   Found.   " 74.8, " 8.0

Levo-n-Propylphenylcarbinol Acetate—15 gm. of *n*-Propyl-phenylcarbinol, $[M]_D^{24} = -22.1°$ (homogeneous), were treated with 30 gm. of acetic anhydride as described for methylphenyl-carbinol acetate. B. p. 134° at 30 mm. Yield 17 gm. $D \frac{22}{4} = 1.000$.

$$[\alpha]_D^{24} = \frac{-10.58° \times 100}{1 \times 20 \times 0.960} = -55.1° \text{ (benzene)}$$

$$[\alpha]_D^{22} = \frac{-57.32°}{1 \times 1.000} = -57.32°; [M]_D^{22} = -110.11° \text{ (homogeneous)}$$

Calculated maximum: $[M]_D^{22} = -178°$ (homogeneous).

     4.066 mg. substance: 11.270 mg. CO_2 and 3.045 mg. H_2O
                $C_{12}H_{16}O_2$. Calculated.   C 74.9, H 8.4
             192.13   Found.   " 75.6, " 8.4

Levo-n-Butylphenylcarbinol Acetate—10 gm. of *n*-butylphenyl-carbinol, $[M]_D^{24} = -13.14°$ (homogeneous), were treated with 20

gm. of acetic anhydride.   B. p. 140° at 20 mm.   Yield 12 gm.
D $\frac{22}{4}$ = 0.988.

$$[\alpha]_D^{24} = \frac{-7.02°}{1 \times 20 \times 0.940} = -37.3° \text{ (benzene)}$$

$$[\alpha]_D^{24} = \frac{-37.50°}{1 \times 0.988} = -37.9°; [M]_D^{24} = -78.07° \text{ (homogeneous)}$$

Calculated maximum: $[M]_D^{24} = -163°$ (homogeneous).

3.521 mg. substance: 9.820 mg. CO_2 and 2.975 mg. H_2O
$C_{13}H_{18}O_2$.   Calculated.   C 75.7, H 8.8
206.14   Found.   " 76.0, " 9.4

A CONTRIBUTION TO THE CHEMISTRY OF LACTO-
BACILLUS ACIDOPHILUS

I. THE OCCURRENCE OF FREE, OPTICALLY ACTIVE, DIHYDROXY-
STEARIC ACID IN THE FAT EXTRACTED FROM
LACTOBACILLUS ACIDOPHILUS

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INTRODUCTION

Lactobacillus acidophilus has been extensively cultivated during the past several years for the preparation of acidophilus milk and other products intended for human consumption. A recent compilation of the acidophilus literature (1) shows that many investigations dealing with the bacteriology and therapeutic application of the bacillus have been published, but that the chemistry of the organism has been entirely neglected. In view of this fact, it seemed pertinent to undertake a study of the chemical compounds contained in the bacillus, and we have accordingly started an investigation which concerns itself first of all with the lipid fractions of *Lactobacillus acidophilus*.

The organism used in our experiments was grown in the Mulford Biological Laboratories, Sharp and Dohme, on a standard culture medium which contains trypsin-digested skim milk, peptone, dextrose, dextrin, and tomato juice. The bacteria were collected in large supercentrifuges, washed with water, and stirred into a large volume of denatured alcohol. It is probable, therefore, that the mass of moist bacteria contained certain constituents, including a small amount of fat, which were derived from the culture medium. This condition will naturally complicate the interpretation of the chemical analyses because we have no assurance that all of the compounds isolated have been synthesized by the bacteria. While this point must be borne in mind, we believe,

nevertheless, that by far the greater part of the lipid substances are of bacterial origin.

The bacterial cells were extracted by methods similar to those used in this laboratory in the investigations dealing with the pathogenic acid-fast bacilli (2). The fat obtained on evaporation of the solvent was found to contain a large proportion of free fatty acids. We wish to report at this time upon the isolation of an optically active dihydroxystearic acid which was found to be present in the free state in the crude fat.

The natural occurrence of dihydroxystearic acid in fats is extremely rare. It was shown by Juillard (3), however, that about 1 per cent of dihydroxystearic acid can be isolated from the mixed fatty acids which are obtained on the saponification of castor oil. A number of isomeric dihydroxystearic acids prepared by oxidation of unsaturated C_{18} acids are mentioned in Lewkowitsch (4), Grün (5), and other texts, but these compounds differ in melting point from the natural acid obtained from castor oil.

It may be of interest in this connection to recall that Schreiner and Shorey (6) and Schreiner and Lathrop (7) showed some years ago that a dihydroxystearic acid, melting point 98–99°, was widely distributed in soils where it was present to the extent of about 50 parts per million. The acid was found to be toxic to plants, and it was believed that it was one cause of the low fertility of certain poor soils. The opinion was expressed that the dihydroxystearic acid might be formed by molds which flourished in this type of soil. It may be possible, however, that the dihydroxystearic acid found by the investigators mentioned above had been formed by certain soil bacteria.

The properties of the acidophilus dihydroxystearic acid differ from those of other known acids of this type. The acid melts at 106–107° and is dextrorotatory; the highest specific rotation observed was +7.78°. The acid is very easily racemized and contact with boiling solvents during the purification caused a marked decline in optical rotation. After the acid had been boiled with dilute alkali, it was found to be optically inactive. On treatment with boiling hydriodic acid, the dihydroxystearic acid was converted into stearic acid.

The extraction of the bacteria and the isolation of the fat fractions will be described in detail in a later publication. It will suffice to state here that the fat was obtained by exhaustive extraction of the bacterial cells with a mixture of alcohol and ether. The solvents were removed by distillation under reduced pressure and the residue extracted with ether. The ethereal solution was dried with sodium sulfate, filtered, and the solvent distilled off. The crude fat weighed about 260 gm. and had a high acid number. It was treated with absolute ether when a portion of the material was found to be insoluble and was filtered off and washed with ether.

The crude substance, after it had been dried *in vacuo*, formed a red-colored amorphous powder which weighed 6.8 gm., corresponding to about 2.6 per cent of the fat. It was insoluble in water, but very soluble in methyl and ethyl alcohol. The alcoholic solutions deposited, on cooling, small aggregates of not definitely crystalline material. The substance dissolved in warm chloroform, and, on cooling, somewhat indistinct needle-shaped crystals separated. It dissolved in warm benzene and the solution solidified on cooling, being completely filled with very small amorphous particles. In hot ethyl acetate the substance dissolved slowly, and, on cooling, there separated a voluminous precipitate consisting of rather ill defined needle-shaped crystals. All of the solutions were of bright red color.

For purification the substance was dissolved in alcohol, treated once with norit, and filtered. The colorless filtrate was evaporated to dryness under reduced pressure and the residue was treated with about 400 cc. of hot ethyl acetate. A slight amount of insoluble matter was filtered off, and, as the solution cooled, a white crystalline product separated. The latter was filtered off, washed with ethyl acetate, and dissolved in about 40 cc. of warm alcohol. Nothing separated from this solution at room temperature, but, on cooling in ice water, colorless globular particles were precipitated. The product, after it had been filtered off, was dissolved in 100 cc. of alcohol and the clear solution diluted with 150 cc. of warm water. On cooling slowly, the substance separated, on scratching, in the form of colorless, fine needles. The crystals were filtered off, washed with dilute alcohol, and dried *in vacuo*.

When heated in a capillary tube, the substance did not show a sharp melting point. It softened at 105° and melted at 108°.

The substance was free from ash, sulfur, phosphorus, nitrogen, and halogen. It dissolved in cold concentrated sulfuric acid, giving a colorless solution, and, on dilution with water, a white precipitate separated. The dilute alcoholic solution of the substance showed a distinctly acid reaction on litmus paper.

Rotation—0.3338 gm. of substance dissolved in methyl alcohol and made up to 10 cc. gave in a 1 dm. tube a reading of +0.26° at 22°; hence, $[\alpha]_D^{22} = +7.78°$.

For further purification, the substance was recrystallized from hot alcohol by the addition of water. The product which separated on cooling was filtered off, washed with dilute alcohol, and dried *in vacuo*. It was then dissolved in hot ethyl acetate, the solution filtered and allowed to cool when fine, not distinctly crystalline particles separated. After the precipitate had been filtered off, washed with ethyl acetate, and dried *in vacuo*, it weighed 3.84 gm. The substance melted sharply at 106–107°, solidified at 101°, and remelted at 106–107°.

Rotation—0.4448 gm. of substance dissolved in methyl alcohol and made up to 10 cc. gave in a 1 dm. tube a reading of +0.13° at 23°; hence, $[\alpha]_D^{23} = +2.92°$.

Analysis—The product was dried at 60° *in vacuo* over dehydrite, but there was no loss in weight.

0.1288 gm. substance: 0.1304 gm. H_2O and 0.3197 gm. CO_2

$C_{18}H_{38}O_4$ (316). Calculated. C 68.35, H 11.39
Found. " 67.69, " 11.32

Titration—0.3385 gm., 0.4332 gm. of the acid dissolved in neutral alcohol, with phenolphthalein as indicator, required 9.73 cc., 12.38 cc. of 0.1 N alcoholic KOH.

$C_{18}H_{38}O_4$. Mol. wt. calculated, 316; found, 347, 349

The silver salt was prepared by adding silver nitrate to a neutral alcoholic solution of the potassium salt, when a white amorphous precipitate separated. The latter was filtered off, washed with dilute alcohol and with alcohol, and dried *in vacuo*.

Analysis—The salt was ignited in a porcelain crucible and the residue of metallic silver weighed.

0.3291 gm. substance: 0.0823 gm. Ag

$C_{18}H_{35}O_4Ag$ (422.88). Calculated, Ag 25.51; found, Ag 25.00

The composition of the free acid and of the silver salt is in approximate agreement with the calculated values for dihydroxy-stearic acid, but the molecular weight found by titration is too high.

Preparation of Acetyl Derivative

The acid, 1.33 gm., was dissolved in 12 cc. of pyridine and 2 gm. of acetic anhydride were added. A slight precipitate which separated was dissolved by warming and the solution allowed to stand overnight. An additional 2 gm. of acetic anhydride were then added. The solution was warmed and allowed to stand at room temperature for several hours. The reaction mixture was then poured into dilute hydrochloric acid, when an oil separated which did not solidify on cooling. The acetyl derivative was extracted with ether and the ethereal solution, after it had been washed thoroughly with water, was dried over sodium sulfate. The drying agent was filtered off and washed with ether, after which the ether was distilled off. The residue was a thick, colorless oil which did not solidify on cooling at 0°. The yield was 1.65 gm. For analysis the oil was dried to constant weight at 60° *in vacuo* over dehydrite.

The acetyl value was determined by saponifying the acetyl derivative with 0.1 N alcoholic potassium hydroxide and titrating the excess of alkali with 0.1 N hydrochloric acid, with phenolphthalein as indicator.

Analysis—0.4792 gm., 0.3824 gm. substance required 24.35 cc., 19.29 cc. of 0.1 N alcoholic KOH.

$C_{18}H_{34}O_4$ (CH$_3$CO)$_2$ (400). Calculated. CH$_3$COOH 30.00
Found. " 30.48, 30.12

The results are in agreement with the calculated values for two acetyl groups.

The balance of the acetyl derivative was saponified. All of the saponification mixtures were combined and united with other solutions obtained in titrating the acid. The mixed solutions were concentrated by distillation, diluted with water, and acidified with hydrochloric acid. The precipitate which separated was filtered off, washed with water until it was free from hydrochloric acid, and dried *in vacuo*. The acid thus recovered was used for the preparation of the barium salt.

Preparation of Barium Salt

The recovered acid was dissolved in alcohol and the solution neutralized with potassium hydroxide, with phenolphthalein as indicator. The solution was diluted with an equal volume of water when a slight cloudiness was produced which was removed by treatment with norit. The clear filtrate was mixed with a slight excess of barium chloride when the barium salt separated as an amorphous precipitate. The latter was filtered off, washed thoroughly with water, and dried *in vacuo.* The amorphous barium salt was dissolved in boiling 60 per cent alcohol, 1 gm. of the salt requiring about 300 cc. The solution was filtered. As the solution cooled, long silky needles separated. The crystals were filtered off, washed with dilute alcohol, alcohol, and ether, and finally dried in a vacuum desiccator. The product formed a snow-white felted mass. Heated in a capillary tube, the substance melted at 208–209°. There was no loss in weight on drying at 105° *in vacuo.*

Analysis—0.1994 gm. substance: 0.0506 gm. $BaCO_3$. The barium carbonate was dissolved in dilute hydrochloric acid and the solution precipitated with sulfuric acid gave 0.0601 gm. $BaSO_4$.

$(C_{18}H_{35}O_4)_2Ba$ (767.4). Calculated.   Ba 17.90
                    Found (as barium carbonate).   " 17.66
                            "   ("   "   sulfate).   " 17.73

Regeneration of Free Acid

The remaining portion of the barium salt was suspended in alcohol, acidified with dilute hydrochloric acid, and the mixture warmed when a nearly clear solution was obtained. The solution was filtered and the clear filtrate diluted with water. The free acid which separated was filtered off, washed thoroughly with water, and redissolved in alcohol. The clear solution was diluted with warm water until it turned slightly cloudy. On cooling and standing for some time, the acid separated in colorless globular particles. The substance was filtered off, washed with dilute alcohol, and dried *in vacuo.* The snow-white powder weighed 1.5 gm. Heated in a capillary tube, the substance melted at 92–93°.

The acid was then dissolved in 50 cc. of hot ethyl acetate, and, as the solution cooled, irregular colorless particles separated.

The product was filtered off, washed with ethyl acetate, and dried *in vacuo*. The snow-white powder, weighing 0.9 gm., melted at 106–107°, solidified at 101°, and remelted at 106–107°. In methyl alcoholic solution the acid was then optically inactive. For analysis the acid was dried at 60° *in vacuo* over dehydrite, but there was no loss in weight.

Titration—0.3115 gm. of substance dissolved in 50 cc. of neutral alcohol, with phenolphthalein as indicator, required 9.68 cc. of 0.1 N alcoholic KOH.

$C_{18}H_{36}O_4$. Mol. wt. calculated, 316; found, 321
0.1063 gm. substance: 0.1075 gm. H_2O and 0.2674 gm. CO_2
$C_{18}H_{36}O_4$ (316). Calculated.   C 68.35, H 11.39
Found.   " 68.60, " 11.31

The observations on the optical rotation would indicate that the acid is very easily racemized. The specific rotation of the acid after three recrystallizations was +7.78°, while after two further recrystallizations the rotation had fallen to +2.92°. The final purified acid, which had been boiled with dilute alkali, was optically inactive.

Reduction of Dihydroxystearic Acid to Stearic Acid

For the reduction of the hydroxy groups, 1 gm. of the acid was refluxed for 3 hours with 20 cc. of acetic anhydride, 10 cc. of hydriodic acid (specific gravity 1.70), and 1 gm. of red phosphorus. After the reaction mixture had cooled, it was diluted with water when a black, tarry oil separated. The liquid was evaporated under reduced pressure nearly to dryness and the black residue dissolved in alcohol. The excess of red phosphorus was filtered off and washed with alcohol, after which the dark brown filtrate was acidified with glacial acetic acid and refluxed for 1½ hours with zinc dust when a colorless solution resulted. The excess of zinc was filtered off and washed with alcohol. The clear filtrate was concentrated to a volume of 40 cc., diluted with water when an oil separated which was extracted with ether. The ether was distilled off and the residue refluxed for 1 hour with 5 per cent alcoholic potassium hydroxide. The saponification mixture, after it had been diluted with water, was extracted with ether and then acidified with hydrochloric acid. The fatty acid which sepa-

rated was extracted with ether, and, after the ethereal solution had been washed with water, it was filtered and the ether evaporated. The snow-white crystalline residue, which weighed 0.8 gm., was dissolved in 10 cc. of warm methyl alcohol. As the solution cooled, large, irregular, colorless plates separated. After the mixture had been cooled in ice water, the crystals were filtered off, washed with cold methyl alcohol, and dried *in vacuo.* The crystals weighed 0.7 gm. and melted at 71–72°. There was no depression of the melting point when some of the substance was mixed with pure stearic acid which melted at 71–72°.

Titration—0.4639 gm. of the acid dissolved in neutral alcohol, with phenolphthalein as indicator, required 16.29 cc. of 0.1 N alcoholic KOH.

$C_{18}H_{36}O_2$. Mol. wt. calculated, 284; found, 284.7
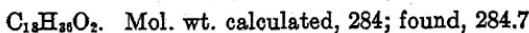
It is evident from the properties and constants of the substance that it was pure stearic acid which had been formed by reduction of the dihydroxystearic acid.

We desire to express our thanks to the Mulford Biological Laboratories, Sharp and Dohme, for supplying *Bacillus acidophilus* in quantity.

SUMMARY

The crude fat, extracted by alcohol and ether, from *Lactobacillus acidophilus* contains an appreciable amount of free dihydroxystearic acid, $C_{18}H_{36}O_4$.

The acid is dextrorotatory, but it is very easily racemized. The highest observed rotation was +7.78°, but, after the acid had been boiled with dilute alkali, it was found to be optically inactive.

The acid crystallizes in somewhat indistinct needles from ethyl acetate and melts at 106–107°. The barium salt crystallizes from 60 per cent alcohol in long, silky needles and melts at 208–209°.

When the acid is reduced with hydriodic acid, it is converted quantitatively into stearic acid.

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THE RELATIONSHIP BETWEEN CHEMICAL STRUCTURE AND PHYSIOLOGICAL RESPONSE

II. THE CONJUGATION OF HYDROXY- AND METHOXYBENZOIC ACIDS*

BY ARMAND J. QUICK

WITH THE TECHNICAL ASSISTANCE OF MARY A. COOPER

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Few studies have been made on the conjugation of *p*- and *m*-hydroxybenzoic acids. Baumann and Herter (1) reported that both acids caused an increase in the output of ethereal sulfates when ingested by man or by the dog. They concluded that these acids are in part combined with sulfuric acid and in part with glycine. They succeeded in isolating from the urine *p*-hydroxyhippuric acid and also an impure preparation of *m*-hydroxyhippuric acid. Schotten (2) also isolated *p*-hydroxyhippuric acid, and he found that after eating 26 gm. of *p*-hydroxybenzoic acid in 24 hours, 35 per cent was excreted unchanged and 16 per cent was combined with glycine. Sherwin (3) failed to find any conjugation of *p*-hydroxybenzoic acid in the monkey; and Power and Sherwin (4) reported similar results when the compound was fed to man.

o-Hydroxybenzoic acid (salicylic acid) has been extensively studied. The comprehensive review of Hanzlik (5) covers the literature up to 1926. There is much conflicting evidence concerning the conjugation of salicylic acid with glycine. Hanzlik (6), after using every method described for the isolation of salicy-

luric acid, failed to find any conclusive evidence of its presence in the urine. The writer (7) likewise, could find no salicyluric acid in the urine of dogs following the feeding of salicylic acid. This finding is in conformity with the rule that the conjugation of glycine with a carboxyl group attached directly to an aromatic ring is greatly inhibited by substitution in the ortho position.

The conjugation of salicylic acid with glycuronic acid was studied by Baldoni (8) who reported a glycuronic acid derivative of salicylic acid, but in a later paper he retracted his claim (9). Neuberg (10) found that when salicylic acid was fed to dogs, the urine showed marked reducing powers, but he failed to isolate a glycuronic acid conjugate. In a recent paper, the writer (7) showed that all three of the hydroxybenzoic acids in the dog are conjugated through their carboxyl group with glycuronic acid; and he expressed the opinion that the hydroxyl group may perhaps also undergo conjugation. A more comprehensive study was therefore deemed desirable, but an immediate attempt to undertake this was not feasible, since these hydroxy acids present a more complex problem than those substituted benzoic acids which have physiologically inactive groups. New procedures and new analytical methods had to be developed or adapted for this investigation. In this paper these new methods and a more complete study of the conjugation of the hydroxybenzoic acids and several closely related compounds are presented.

EXPERIMENTAL

The experiments on dogs were carried out the same as in former studies. The acid studied was neutralized and incorporated with the diet consisting of casein, sucrose, lard, and bone ash. In the human studies the routine followed was as follows: Breakfast, consisting of a cup of coffee and a cruller, was eaten at 8.00 a.m. 1 hour later the acid which was neutralized with sodium hydroxide was taken in 25 cc. of water followed by 75 cc. of milk. This procedure prevented the nausea and headache which frequently resulted when the drug was taken in the fasting state. The buffer action of the milk tends to reduce the gastric irritation which these compounds are apt to produce. Hourly specimens of urine were collected.

Analytical Methods

The author's methods for determining hippuric acid and gly-curonic acid monobenzoate were applied as in the previous study (7) to the corresponding conjugated products of the substituted benzoic acids with satisfactory results. The procedure developed for the determination of uncombined substituted benzoic acids was found applicable to all the compounds studied except *p*-hydroxybenzoic acid. This compound is too difficultly soluble in toluene to make a complete extraction feasible unless the amount of free acid present is very small.

Bromination Method of Day and Taggard (11) Applied to the Determination of Free and Total Hydroxybenzoic Acids—This excellent method was found very valuable for the determination of the hydroxybenzoic acids and their derivatives. When the method is combined with the author's ether extraction procedure, it offers a satisfactory and simple means for determining the hydroxybenzoic acids and their derivatives in human urine, and with minor modifications should become applicable for determining various other phenolic compounds. The procedure as employed for human urine is as follows: 5 to 10 cc. of urine, to which 1 cc. of 5 N sulfuric acid and 2 drops of 10 per cent sodium tungstate are added, are extracted in a continuous extractor with ether for 90 minutes. The ether is distilled off, the residue is dissolved in 5 cc. of hot water and titrated with 0.1 N sodium hydroxide, phenolphthalein being used as indicator. From the titration the approximate quantity of substance extracted is calculated. If it exceeds 0.1 gm., the solution should be diluted to a definite volume and an aliquot containing approximately 0.1 gm. of the substance taken. The neutralized solution or aliquot is transferred to an iodine flask and diluted to 75 cc. 25 cc. of 0.2 N bromate solution (75 gm. of KBr and 5.6 gm. of KBrO₃ per liter) are added. The solution is acidified with 5 cc. of concentrated hydrochloric acid and shaken for 1 minute. After 30 minutes the flask is cooled under the tap and 5 cc. of 40 per cent potassium iodide are poured into the trough of the flask. By partly dislodging the stopper the solution is drawn into the flask. An ordinary 500 cc. glass-stoppered bottle serves equally as well as an iodine flask, provided one cools the reacting solution well before adding the potassium

iodide. The liberated iodine is titrated with 0.1 N sodium thio-
sulfate, starch solution being used as indicator. The amount of
bromine taken up by the compound is calculated from the differ-
ence between the number of cc. of thiosulfate solution required in
the titration of the sample, and the titration of the blank with 25
cc. of 0.2 N bromate solution. From the bromine consumed,
the quantity of substance in the sample can be calculated. The
bromine equivalents of the various compounds studied are:

p-Hydroxybenzoic acid................	3 Br$_2$	p-Hydroxyhippuric acid..............	2 Br$_2$
m-Hydroxybenzoic acid................	3 "	m-Hydroxyhippuric acid..............	3 "
o-Hydroxybenzoic acid................	3 "		
p-Methoxybenzoic acid................	1 "	p-Methoxyhippuric acid..............	1 "
m-Methoxybenzoic acid................	1-2 "	m-Methoxyhippuric acid..............	1-2 "
o-Methoxybenzoic acid................	1 "		
p-Hydroxybenzoic acid-diglycuronic acid................	3 "		

The m-hydroxy- and m-methoxybenzoic acids will take up 1 atom
of bromine immediately and a 2nd atom slowly.

The amount of acid excreted free is determined from the differ-
ence between the total bromine consumed and the calculated
amount of bromine taken up by the hippuric acid. Of course
this requires a determination of hippuric acid on a second sample.

The method as outlined is not applicable to urines containing
appreciable amounts of conjugated glycuronic acids, since these
are partially extracted by ether. To determine the total quantity
of a hydroxybenzoic acid present in urine containing glycuronic
acid one proceeds as follows: 5 cc. of urine are mixed with 5 cc.
of concentrated hydrochloric acid and the mixture boiled for 90
minutes in a 125 cc. Erlenmeyer flask provided with an air con-
denser. On cooling, the solution is transferred to a continuous
extractor and extracted with ether for 60 minutes. The extract,
after removal of the ether, is neutralized with 0.1 N sodium hy-
droxide and the bromination carried out as described before.

Isolation of p-Hydroxybenzoic Acid-Diglycuronic Acid (Glycuronic Acid-p-Glycuronidebenzoate)—5 gm. of neutralized p-hydroxybenzoic acid are incorporated in the diet and fed to a dog. In order to keep the volume of urine as low as possible the dog is catheterized immediately after feeding and again at the end of 8 hours. Since most of the conjugated acid is excreted during the first 6 to 8 hours, collection after this period is unprofitable. The urine is slightly acidified with acetic acid, and lead acetate added until no further precipitation occurs. The precipitate which carries down most of the coloring matter is filtered off; the filtrate is made acid to Congo red and extracted with repeated portions of butyl alcohol. The partition coefficient of the compound between water and butyl alcohol is approximately 1. The extraction can be followed polariscopically. Extraction in a continuous extractor under reduced pressure offers a more efficient but technically more difficult procedure. When the extraction is about 75 per cent completed, the butyl alcohol fractions are united and distilled under reduced pressure. The residue which consists of a crystalline mass is recrystallized from hot water. By further concentration of the mother liquor, the yield can be increased. The crystalline product, which after one recrystallization is pure white, is extracted with 100 volumes of ether to remove any traces of hippuric acid and free p-hydroxybenzoic acid. About 1.3 gm. of product can be obtained after feeding 5 gm. of p-hydroxybenzoic acid. On analysis the pure product corresponds to 2 molecules of glycuronic acid and 1 of p-hydroxybenzoic acid.

Analysis—Titration with 0.1 N sodium hydroxide; sample 0.1 gm. Found 4.1 cc.; calculated 4.1 cc.

Bromination (30 minutes); sample 0.1 gm. Found 0.105 gm.; calculated 0.098 gm.

Glycuronic acid in ester linkage (Shaffer-Hartmann method directly); sample 0.1 gm. Found 0.0378 gm.; calculated 0.0395 gm.

Glycuronic acid, total; after 15 minutes hydrolysis with 1.0 N hydrochloric acid; sample 0.1 gm. Found 0.072 gm.; calculated 0.079 gm.

The slightly low results for total glycuronic acid appear to be due to a partial destruction of the compound during hydrolysis. The low results for glycuronic acid in ester linkage can be explained on the basis that the reducing action of conjugated glycuronic acid is less than an equivalent amount of the free acid, as has been

previously observed in the case of glycuronic acid monobenzoate (12).

p-Hydroxybenzoic acid-diglycuronic acid crystallizes in the form of fine needles which have a tendency to be grouped in rosettes. The compound is stable; it is readily soluble in hot water but sparingly in cold water. At 20° about 1 gm. is dissolved in 100 cc. of water. The compound melts with decomposition when heated slightly over 200°. The optical rotation of a 1 per cent solution is $[\alpha]_D^{20} = -15°$ to $-16°$. In alkaline solution it shows mutarotation, but the change of rotation is much slower than that of glycuronic acid monobenzoate.

The probable structure of the compound is
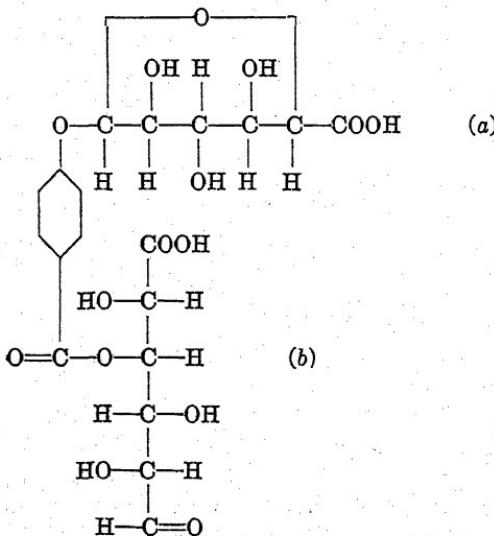
The exact hydroxyl group of glycuronic acid (b) to which the aromatic carboxyl group is united is not known. It is probable that the glycuronic acid (b) has a pyranose structure. On examining the formula as given, it can be seen that the compound exhibits the properties of both the glycosidic and the ester type of glycuronic acid. The glucosidic linkage is stable, resists alkaline hydrolysis, but is split when subjected to boiling with 1.0 N hydrochloric acid. The glycuronic acid (b) which is in ester linkage with the p-hydroxybenzoic acid has the same properties previously noted for glycuronic acid monobenzoate. By virtue of its free

aldehyde group, it reduces the common sugar reagents directly and shows mutarotation. The action of phenylhydrazine, hydrocyanic acid, and other reagents used in carbohydrate studies has not been investigated. In view of the ease with which the compound can be prepared, it should become very useful for theoretical studies on the molecular structure of carbohydrates.

Attempts to Isolate m- and o-Hydroxybenzoic Acid-Diglycuronic Acid—Although *m*- and *p*-hydroxybenzoic acids behave very much alike in the organism of the dog, as indicated by quantitative analysis for glycuronic acid and by the optical rotation of the urine, efforts to isolate the diglycuronic acid derivative of the meta compound failed. The conjugated product is formed, can be extracted with butyl alcohol, but will not crystallize. A sample of the gum was analyzed for glycuronic acid before and after acid hydrolysis, and it was found that the hydrolysis doubled the amount of glycuronic acid. On feeding salicylic acid to a dog, conjugation with glycuronic acid results, but the amount excreted is much smaller than occurs after feeding either the meta or para acid. Thus, after feeding 3.5 gm. of salicylic acid to a 10 kilo dog, 0.67 gm. of glycuronic acid was found in the first 6 hour urine specimen by directly applying the Shaffer-Hartmann method, whereas after acid hydrolysis the glycuronic acid content was found to be 1.16 gm. On extracting the urine with butyl alcohol, a crystalline product was obtained which reduced Benedict's solution.

Isolation of Hydroxy- and Methoxyhippuric Acids—The various hippuric acids derived from substituted benzoic acids are best isolated from human urine. About 3 to 5 gm. of the neutralized acid and 3 gm. of glycine are ingested. The urine voided during the first 5 hours is collected, made acid to Congo red with sulfuric acid, and extracted in a continuous extractor for 90 minutes. The extract, after the ether is distilled off, is treated with boiling toluene to remove any free acid. In the case of *p*-hydroxybenzoic acid which is difficultly soluble in that solvent, it is best to extract the dry crystalline mass with a small amount of ether which will readily remove the free acid without any appreciable loss of the hippuric acid. The crystalline residue remaining is recrystallized from hot water with a small amount of decolorizing charcoal. The compounds prepared are given in Table I.

The bromination of the hydroxy- and methoxyhippuric acids

shows great variation. *p*-Hydroxyhippuric acid will take up 2 atoms of bromine, but the carboxyl group which is linked with glycine remains intact, whereas in the case of the meta acid the carboxyl group is split off and 3 atoms of bromine will enter the molecule. *p*-Methoxyhippuric acid will take up only 1 atom of bromine while *m*-methoxyhippuric acid will take up 1 atom readily and a 2nd one slowly.

TABLE I

Hydroxy- and Methoxyhippuric Acids

Compound	M.p. uncorrected	Titration*		Glycine*		Bromine*	
		Found	Calculated	Found	Calculated	Found	Calculated
	°C.	cc.	cc.	gm.	gm.	gm.	gm.
p-Hydroxyhippuric acid......	227	5.80†	5.15	0.0375	0.0384	0.161	0.164
m-Hydroxyhippuric "	182–183	5.30	5.15	0.0375	0.0384	0.235	0.246
p-Methoxyhippuric "	170	4.70	4.80	0.0337	0.0358	0.078	0.076
m-Methoxyhippuric "	122	4.70	4.80	0.0345	0.0358		

* Sample 0.1 gm.

† Although *p*-hydroxybenzoic acid was obtained in a high state of purity, as indicated by the constancy of the melting point and by various analyses, the titration, nevertheless, was consistently higher than the calculated value.

DISCUSSION

The conjugation of *p*-hydroxybenzoic acid with 2 molecules of glycuronic acid, one of which is in ester linkage, the other in glucoside union, presents a unique demonstration of the synthetic power of the organism. Both types of conjugation take place practically simultaneously, and it is reasonable to suspect that the site of these syntheses is the liver, since the production of such a relatively large quantity of glycuronic acid requires an ample and readily available source of carbohydrate such as is present in that organ. Although both molecules of glycuronic acid probably have the same precursor, their conjugation in two distinctly different types of linkage must undoubtedly require two separate mechanisms.

While the diglycuronic acid compound is the main conjugation product of *p*-hydroxybenzoic acid when fed to dogs, other com-

pounds are also found. Theoretically, six different compounds are possible, for the hydroxy group may remain free or be combined with glycuronic acid or sulfuric acid, while the carboxyl group may be uncombined or be united with glycine or glycuronic acid. In the previous study as well as in the present, the author has shown that a small fraction of the p-hydroxybenzoic acid is excreted as the corresponding hippuric acid. It is also possible that a small fraction is excreted as an ethereal sulfate although the data of Baumann and Herter (1) are not convincing. Quantitatively the glycuronic acid conjugations are, however, most important. There are indications that a fraction of the p-hydroxybenzoic acid is excreted with glycuronic acid attached only to the hydroxyl

TABLE II

Fate of p-Hydroxybenzoic Acid and p-Hydroxybenzaldehyde in the Dog

Compound	Urine volume	α_D 1 dm. tube	p-Hydroxybenzoic acid excreted			
			Combined with			Total
			Glycine	Glycuronic acid		
				As glucoside	As ester	
	cc.		gm.	gm.	gm.	gm.
p-Hydroxybenzoic acid*	71†	−1.30	0.31	1.18	1.31	2.10
p-Hydroxybenzaldehyde‡	135	−2.00	0.48	1.12	1.07	1.90

* 3.5 gm. of p-hydroxybenzoic acid were fed.
† Excretion during the first 6 hours.
‡ 3.0 gm. of p-hydroxybenzaldehyde were fed.

group since the rotation of the urine is much more levorotatory than can be accounted for by the diglycuronic acid compound alone. Furthermore, when p-hydroxybenzaldehyde is fed to a dog, a smaller amount of the diglycuronic acid compound is excreted and the levorotation of the urine is markedly increased (Table II). Apparently when p-hydroxybenzaldehyde reaches the liver through the portal circulation, the hydroxy group becomes conjugated with glycuronic acid, but the aldehyde remains unchanged. Later, outside the liver the aldehyde radical is oxidized to an acid and is excreted as such, except for the portion that again passes through the liver and there is united with a 2nd molecule of glycuronic acid.

The conjugation of *m*- and *o*-hydroxybenzoic acids is very similar to that of the para compound. After feeding either acid, it will be found that the reducing action of the urine after acid hydrolysis is practically doubled, which is definite evidence that the urine contains the diglycuronic acid derivative. The ortho acid, in contrast to the meta and para isomers, is excreted much more slowly.

When 3 gm. of *p*-hydroxybenzoic acid-diglycuronic acid were ingested by man, *p*-hydroxybenzoic acid was slowly eliminated

TABLE III

Fate of p-Hydroxybenzoic Acid-Diglycuronic Acid in Man*

Time	Urine volume	α_D†	Excretion of *p*-hydroxy-benzoic acid	
			Free	With glycine
	cc.		*gm.*	*gm.*
9.00 −10.00 a.m	27‡	−0.20	0.06	0.06§
10.00 −11.00 "	37	−0.15	0.06	0.10
11.00 −12.00 "	37	−0.12	0.06	0.06
12.00 a.m.− 1.00 p.m	37	−0.18	0.06	0.06
Blank	42	−0.13	0.02	0.02

* 3 gm. of *p*-hydroxybenzoic acid-diglycuronic acid were ingested at 9.00 a.m. This is equivalent to 0.8 gm. of *p*-hydroxybenzoic acid.

† Rotation observed in a 1 dm. tube. A small levorotation is found in normal urine.

‡ No reduction of Benedict's solution by any specimen of urine.

§ Both free *p*-hydroxybenzoic acid and *p*-hydroxyhippuric acid were isolated. 20 mg. of the latter compound were identified by its characteristic long rectangular crystals, its solubility behavior, and its melting point 220°. (The pure compound melts at 227°.)

partly as the free acid and partly combined with glycine, but no trace of glycuronic acid could be detected (Table III). This finding is in complete agreement with the author's previous observations that combined glycuronic acids, whether in glucoside or ester linkage, can be and are oxidized in the body. It is obvious that if *p*-hydroxybenzoic acid is partly conjugated in the human organism with glycuronic acid, no evidence of it can necessarily be obtained from the urine, since the glycuronic acid is again oxidized before excretion occurs.

The conjugation of the three hydroxybenzoic acids in man admirably demonstrates the sensitivity of the organism's response to the position of the active group in the benzene nucleus (Table IV). Of the three acids, *m*-hydroxybenzoic acid resembles benzoic acid most closely. This acid is combined mainly with glycine

TABLE IV

*Conjugation of Hydroxy- and Methoxybenzoic Acids in Man**

Time	*p*-Hydroxybenzoic acid		*m*-Hydroxybenzoic acid		*o*-Hydroxybenzoic acid	
	Free	With glycine	Free	With glycine	Free	With glycine
hrs.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
1	0.44	0.27	0.12†	0.50	0.16	0.02
2	0.58†	0.54	0.12†	0.67	0.19	0.02
3	0.29	0.42	0.01†	0.69	0.12	0.02
4	0.00	0.29	0.03	0.33	0.13	0.02

With 3 gm. glycine

1	0.51	0.26	0.13†	0.52	0.15	0.05
2	0.42†	0.60	0.19†	0.93	0.20	0.07
3	0.00	0.60	0.00†	0.70	0.20	0.07
4	0.00	0.30	0.00	0.40		0.07

	p-Methoxybenzoic acid		*m*-Methoxybenzoic acid		*o*-Methoxybenzoic acid	
	With glycuronic acid	With glycine	With glycuronic acid	With glycine	With glycuronic acid	With glycine
1	0.31	0.38	0.00	0.45	†	0.05
2	0.53	0.52	0.11	0.55	†	0.06
3	0.42	0.53	0.11	0.62	†	0.06
4	0.25	0.16		0.66	†	0.21‡

* 3.4 gm. of the hydroxybenzoic acids and 3.7 gm. of the methoxybenzoic acids were taken.

† The urine showed a slight but distinct reduction with Benedict's solution.

‡ 3 gm. of glycine were taken at the end of the 3rd hour.

and only to a small degree with glycuronic acid. Nevertheless, in contrast to benzoic acid, a small but definite fraction is excreted uncombined, especially during the 1st and 2nd hours. *p*-Hydroxybenzoic acid, on the other hand, is excreted partly free and partly combined with glycine. During the early period the free acid con-

stitutes the larger fraction. It is to be noted that the addition of glycine to the diet does not appreciably diminish the amount of free acid, a finding also observed for the meta and ortho acids.

The question arises whether the uncombined acid appearing in the urine traverses the body without undergoing conjugation or whether it is derived from that fraction which is initially combined with glycuronic acid but is subsequently reliberated by the oxidation of the latter compound. The evidence is in favor of the second supposition. In the first place, after eating p-hydroxybenzoic acid a small amount is excreted combined with glycuronic acid, as indicated by the reducing action of the urine. This shows that the human organism can not only conjugate the compound with glycuronic acid, but, moreover, produce enough to exceed the kidney threshold. As pointed out before, when 3 gm. of the diglycuronic acid compound of p-hydroxybenzoic acid are ingested, none of the original compound is excreted. The inability of excess exogenous glycine to reduce materially the output of free acid and to increase the amount of hippuric acid suggests that the carboxyl group must be initially combined presumably with glycuronic acid so that conjugation with glycine is prevented. Finally, one cannot overlook the strange coincidence that when p-methoxybenzoic acid is eaten, the fraction which becomes conjugated with glycine is approximately the same as found for p-hydroxybenzoic acid, and the remaining portion which is combined with glycuronic acid corresponds roughly in quantity to the free acid found in the case of p-hydroxybenzoic acid. The following explanation is offered: The initial conjugation of p-hydroxybenzoic acid and p-methoxybenzoic acid is quantitatively essentially the same except that 2 molecules of glycuronic acid are combined with the former, and only 1 with the latter, because it lacks a free hydroxyl group. The fractions which are combined with glycine are promptly excreted, and similarly the monoglycuronic acid derivative of p-methoxybenzoic acid readily escapes into the urine. The diglycuronic acid compound, on the contrary, is not excreted but undergoes oxidation, resulting in the loss of both molecules of glycuronic acid and the excretion of p-hydroxybenzoic acid in the unconjugated form. An examination of Table IV shows that a similar explanation can be offered for the coincidental correspondence of the glycuronic acid conjugation and the excre-

tion of free acid in the case of *m*-methoxy- and *m*-hydroxybenzoic acids; and of *o*-methoxy- and *o*-hydroxybenzoic acids.

The studies on the excretion of *o*-hydroxybenzoic acid (salicylic acid) can only be considered as preliminary, but nevertheless enough new data have been secured to throw some light on the possible fate of this compound in the body. Strikingly, no conjugated salicylic acid is normally excreted. Occasionally individuals can be found who excrete a small amount combined with glycuronic acid. In agreement with Hanzlik, no salicyluric acid could be found. This is to be expected, since it conforms with the rule that the glycine conjugation with an aromatic carboxyl group is markedly inhibited by ortho substitution. After the ingestion of salicylic acid only the free acid is excreted and at a very slow rate. Less than 0.2 gm. per hour was the rate found for a normal subject. Surprisingly, the literature fails to give any accurate data on the hourly excretion in spite of the extensive use of salicylates in therapy.

As in the case of *p*-hydroxybenzoic acid, the excretion of free salicylic acid does not necessarily mean that the drug has traversed the body without undergoing conjugation. On the contrary, there is much more evidence that salicylic acid like *p*-hydroxybenzoic acid is combined with glycuronic acid and there is a possibility that the therapeutic action of salicylic acid may be dependent upon the metabolism of the glycuronic acid-salicylic acid compound. This problem will be further studied, but even now it can be stated with certainty that any comprehensive study of salicylic acid must include a proper evaluation of the glycuronic acid conjugation.

The study of the conjugation of *p*-methoxybenzoic acid recorded in Table V yields valuable information. In the first place it furnishes definite evidence that the human can readily produce large quantities of glycuronic acid to combine with the carboxyl group of an aromatic acid. The latent ability of the human organism to produce nearly a gm. of glycuronic acid per hour strongly suggests that the importance of glycuronic acid in metabolism is decidedly underestimated. The ease with which the conjugation of *p*-methoxybenzoic acid can be influenced opens up new possibilities in regulating the synthetic processes of the body. In the absence of exogenous glycine, about one-half of the ingested

p-methoxybenzoic acid is combined with glycuronic acid, the remaining moiety with glycine. By feeding *o*-toluic acid with *p*-methoxybenzoic acid, the glycine conjugation is definitely reduced with a concomitant increase in the glycuronic acid synthesis. While the ratio of glycine to the glycuronic acid conjugation is greatly changed, the total amount of acid conjugated per hour remains practically the same, indicating that the capacity of the organism is fairly well fixed. The action of *o*-toluic acid can be explained by assuming that while the compound itself is not to any appreciable extent excreted combined with glycine, it, never-

TABLE V

Repression and Stimulation of Synthesis of Hippuric Acid as Applied to p-Methoxybenzoic Acid

Time	Excretion of *p*-methoxybenzoic acid* combined with					
	Normal		*o*-Toluic acid, 1.0 gm. taken		Excess glycine, 3.0 gm. taken	
	Glycine	Glycuronic acid	Glycine	Glycuronic acid	Glycine	Glycuronic acid
hrs.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
1	0.38	0.31	0.28	0.37	0.75	0.22
2	0.51	0.53	0.32	0.69	1.03	0.40
3	0.50	0.42	0.28	0.63	0.29	0.23
4	0.16	0.25				

* 3.7 gm. of *p*-methoxybenzoic acid were taken in each experiment

theless, seriously interferes with the glycine conjugation, as a result of which the production of *p*-methoxyhippuric acid is delayed, and a greater opportunity is given for the compound to unite with glycuronic acid. The characteristic inhibiting effect of ortho-substituted benzoic acid on the glycine conjugation will be discussed in greater detail in a later paper. By supplying excess glycine, a marked increase in the output of *p*-methoxyhippuric acid can be produced. Thus, by the simple procedure of feeding *o*-toluic acid in one experiment and excess glycine in another, one can produce a difference in the hippuric acid output of over 300 per cent, and a change of 50 per cent in the glycuronic acid conjugation.

New Theory of Detoxication

It has long been recognized that the toxicity of the benzene nucleus is greatly reduced by the introduction of the carboxyl group. Thus, phenol, aniline, and nitrobenzene are converted respectively to the relatively non-toxic hydroxy-, amino-, and nitro-benzoic acids. In fact the introduction of a carboxyl group into benzene itself converts this distinctly toxic substance into harmless benzoic acid, and a compound such as toluene, which can readily be converted to benzoic acid, is practically non-poisonous.

In reviewing the major conjugation processes of the body, one is struck by the fact that the conjugated product is always a stronger acid than the original compound. The ionization constant of benzoic acid is 6.5×10^{-5}, whereas that of hippuric acid is 2.3×10^{-4} and of glycuronic acid monobenzoate 3.0×10^{-3}. Similarly, phenylacetic acid has an ionization constant of 5.6×10^{-5} and phenylaceturic acid of 2.0×10^{-4}. While menthol is a neutral compound, menthol glycuronic acid has a constant of approximately 1×10^{-3}; in fact all the glycuronic acids both of the glucosidic and of the ester type are relatively strong acids. Phenol, a very weakly acidic compound when combined with sulfuric acid gives rise to a strong acid, phenolsulfuric acid; similarly indoxyl is converted to indoxylsulfuric acid. Bromobenzene is converted to p-bromophenyl-mercapturic acid which is presumably a strong acid. Even acetylation will produce a stronger acid, thus p-acetylaminobenzoic acid is stronger than the parent p-amino-benzoic acid.

The question arises whether the production of strong acids by the mechanism of conjugation is merely incidental or whether it constitutes a fundamental factor in the detoxication and excretion of a foreign substance. It should be remembered that benzoic acid is a relatively harmless substance. A nephrectomized dog, when given as much as 3 gm. intravenously shows no toxic symptoms in spite of the fact that the animal has lost the power to synthesize hippuric acid, and even though a portion of the acid is circulating through the body in the uncombined state. Nevertheless, neither man nor the dog will excrete more than a trace of the uncombined acid, and while a curtailment of the supply of glycine or glycuronic acid will delay the excretion of combined ben-

zoic acid, it will not cause the appearance of free benzoic acid in the urine. Therefore, one is led to the conclusion that conjugation may not be primarily a detoxication mechanism in the sense of rendering a toxic group inert, but a means of changing a weak acid which the body cannot excrete to a strong acid which it can eliminate. As further confirmatory evidence, strong acids such as mandelic ($C_H = 4.3 \times 10^{-4}$) and o-nitrobenzoic acid (6.2×10^{-3}) are readily excreted uncombined, indicating that conjugation is not a necessary step in the excretion provided the compound is a sufficiently strong acid. A *priori*, p-hydroxybenzoic acid appears to be an exception to this rule, for although it is a weak acid, it is partly excreted free. There is good evidence to indicate, however, that the free acid found in the urine has not passed through the body unchanged, but is derived from the strongly acidic glycuronic acid conjugate.

SUMMARY

1. The bromination method of Day and Taggard has been adapted for the determination in urine of free and total hydroxybenzoic acids and related compounds.

2. p-Hydroxybenzoic acid-diglycuronic acid (glycuronic acid-p-glycuronide benzoate) has been isolated in pure crystalline form and studied. This compound is the main conjugation product when p-hydroxybenzoic acid is fed to dogs. Evidence has been obtained that o- and m-hydroxybenzoic acids are similarly combined with 2 molecules of glycuronic acid.

3. A method for the isolation of substituted hippuric acids has been described. The following compounds were isolated: p-hydroxyhippuric acid, m-hydroxyhippuric acid, p-methoxyhippuric acid, and m-methoxyhippuric acid.

4. When p-hydroxybenzoic acid-diglycuronic acid is ingested by man, none of the compound is excreted, but p-hydroxybenzoic acid appears in the urine partly free and partially combined with glycine.

5. p-Hydroxybenzoic acid in man is excreted partly uncombined and partly united with glycine, m-hydroxybenzoic acid is conjugated mainly with glycine, while o-hydroxybenzoic acid is eliminated free.

6. p-Methoxybenzoic acid is conjugated with glycine and

glycuronic acid in the approximate ratio of 1:1; m-methoxy-benzoic acid is chiefly combined with glycine; and o-methoxy-benzoic acid is very slowly eliminated conjugated with glycuronic acid and to a very limited degree with glycine.

7. The conjugation of p-methoxybenzoic acid can readily be influenced; by means of o-toluic acid its conjugation with glycine can be greatly depressed, and by means of excess glycine markedly increased.

8. A new theory of conjugation is presented; a fundamental factor in a conjugation process is the conversion of a weak acid, which the body apparently cannot excrete, to a strong acid, which it can eliminate through the kidney.

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THE ANTINEURITIC VITAMIN

III. REMOVAL OF IMPURITIES BY FRACTIONAL PRECIPITATION*

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The modified method of Jansen and Donath (1), although
capable of yielding a crystalline material believed to be the pure
antineuritic vitamin, has two distinctly undesirable features, first
the large number of steps in the purification, and second, the low
yields of many of the procedures; the manipulations are so diffi-
cult to control properly that even an experienced worker is unable
to obtain approximately constant results (Seidell and Birckner
(2)). The highly potent compounds prepared by Van Veen (3)
and by Windaus, Tschesche, Ruhkoff, Laquer, and Schultz (4)
were secured only after elaborate methods of purification, which
involved adsorption on and elution from fullers' earth, purifica-
tion by successive treatments with mercuric sulfate and silver
nitrate, benzoylation, use of gold chloride, and finally precipita-
tion of the antineuritic substance as the picrolonate and as the
hydrochloride.

In our work (5, 6) the object has been to prepare a vitamin B_1
concentrate that may be injected parenterally with complete
safety, and which can be easily prepared in the average chemical
or physiological laboratory. The experimental work to be de-
scribed below deals with modifications of the method previously
recorded (5). In addition, it is concerned with many attempts
further to purify the vitamin concentrate obtained by our carbon
tetrachloride-oxidation technique (6), and with confirmatory

biological tests performed on species of animals other than the pigeon, which is used in our assay method already described (5).

Modifications of Carbon Tetrachloride-Oxidation Technique

Preparation of Crude Concentrate—It seemed desirable to apply the carbon tetrachloride procedure directly to natural sources of the vitamin such as rice polish and yeast without first removing vitamin B_2 (G) by adsorption on fullers' earth (7, 8).

Rice polishings in a large cylindrical vessel were extracted with cold tap water, the supernatant liquid was siphoned off, and the solution concentrated *in vacuo* to a thin syrup. The reaction of the vitamin solution should be adjusted to pH 4 to 5 by hydrochloric acid before the concentration. If the solution is too alkaline, destruction of the vitamin takes place; if too acid, the carbohydrates are caramelized. The syrup is filtered and the volume adjusted so that there is approximately 35 per cent of total solids present. This solution is then further purified by the technique previously described (6). The concentrate so obtained is essentially the same as tikitiki prepared according to the method of Wells (9), except that the carbohydrates of high molecular weight as well as many toxic nitrogenous impurities are destroyed.

Dried brewery yeast was extracted with hot dilute hydrochloric acid at pH 3 to 4, the temperature being maintained at about 90° for 10 minutes in order to coagulate the proteins. The solution was then filtered and concentrated in large evaporating dishes over a free flame. If the solution is sufficiently acid there is no appreciable destruction of the vitamin even when the surface is stirred by a current of warm air from an electric hair drier. The concentrated material was put through the usual carbon tetrachloride technique (5, 6). In our early experiments on yeast, acetic acid was used as the coagulating agent. This treatment resulted in an almost complete destruction of the potency, only about 10 per cent of the vitamin being recovered. The destructive action of acetic acid and especially acetic anhydride on the vitamin is described below in greater detail. Assays for the antineuritic vitamin only (7) have been carried out on these fractions.

Changes in Oxidation Procedure—It has been observed that the

carbon tetrachloride-oxidation technique is capable of very extensive modification without affecting the potency of the vitamin. The ratio of the original vitamin solution to the ethyl alcohol-carbon tetrachloride mixture may vary from 0.5 to 5.0. The acidity of the concentrate may vary from below pH 1 to pH 4; the addition of a few cc. of hydrogen peroxide to the vitamin solution during the removal of the organic solvents and excess hydrochloric acid results in a slightly purer concentrate.

Attempt to Remove Amino Nitrogen during Carbon Tetrachloride Process—Sherman and Whitsitt (10) have shown that the antineuritic vitamin is relatively stable to nitrous acid. On the basis of this work, it seemed desirable to ascertain whether it would be possible to reduce the amount of nitrogenous impurities by treatment with nascent nitrous acid during the carbon tetrachloride-

TABLE I

Treatment of the Vitamin with Nitrous Acid

Experiment No.	NaNO₂	N recovered	Recovery of vitamin
	gm.	*mg.*	*per cent*
1	0.0	110	100
2	0.5	110	100
3	0.625	110	100
4	1.0	110	45
5	2.0	100	45

oxidation process. A fullers' earth eluate containing 8.0 gm. of total solids and 110 mg. of nitrogen was diluted to 25 cc. with water, and then 5 cc. of concentrated hydrochloric acid, 3 cc. of 10 per cent barium chloride, and 200 cc. of a 1:1 mixture of ethyl alcohol-carbon tetrachloride were added. The solution was heated to boiling in the usual apparatus (5, 6) and various amounts of sodium nitrite in a few cc. of water were then introduced (Table I). The reaction was allowed to proceed for 3 hours when 1.5 cc. of 30 per cent hydrogen peroxide were added. The rest of the reaction was carried out in the manner previously described. The results of these experiments, which are summarized in Table I, show that there was no decrease in the total amount of nitrogen present. Although the vitamin potency was not reduced when 0.5 gm. and 0.625 gm. of sodium nitrite were used, increasing the

amount to 1.0 gm. and 2.0 gm. resulted in only 45 per cent of the initial potency being demonstrable at the end of the reaction.

These data can be taken as confirmation of the conclusions of Sherman and Whitsitt (10) that the antineuritic vitamin is resistant to destruction by nitrous acid but succumbs if the conditions are made drastic enough. It is possible, of course, that the destruction is due to oxidation, but in the light of our previous results (6) the first suggestion is more probable.

Purification of Vitamin Concentrate Obtained by Oxidation Procedure—A vitamin concentrate, quite free from inorganic material and carbohydrate gums of high molecular weight, appeared to offer a much better starting point for further purification than any other easily obtained concentrate hitherto described. The guiding principle in almost all our experiments was to use reagents that are easily manipulated, which can be cheaply recovered, and which are non-toxic when present in small amounts.

Dakin and West (11) described an interesting method for the extraction of bases and amino acids as picrates by the use of mixed solvents, even though no obvious formation of a sparingly soluble picrate could be observed. On account of the fact that the antineuritic vitamin behaves as a base, it was hoped that an effective concentration of the active material could be made by this procedure.

A vitamin solution, obtained by the fullers' earth-oxidation technique, was concentrated to dryness to remove all the free hydrochloric acid. The residue was diluted to a 10 per cent solution and an excess of the reagent (picric acid, flavianic acid, and rufianic acid) was slowly poured into the boiling vitamin concentrate. The reaction mixture was allowed to stand overnight at 4°, and if any precipitate appeared, it was filtered off. The precipitate was washed with a little cold dilute reagent and decomposed in the customary manner, with hydrochloric acid and ether. The filtrate was extracted four times with equal volumes of 1:1 butyl alcohol-ether mixture. Each of the three fractions (precipitate, water layer, and solvent layer) was tested for antineuritic potency after removal of solvents and reagents. The results are given in Table II. It will be noticed that only a very small amount of the vitamin was extracted by the solvent in the presence of picric and rufianic acids but that almost all of the potency

appeared in the butyl alcohol-ether layer when flavianic acid was used. Unfortunately, however, a large proportion of the nitrogenous impurities was also extracted by the solvent and therefore no significant concentration of the vitamin took place. It is possible that the poor results which were obtained might be due to the fact that the non-volatile acids present prevented the formation of the basic salts. This idea may be tested by experiments in which the organic acids are neutralized with dilute sodium hydroxide. Such studies are in progress.

In view of the results obtained with the methods of fractionation described above, we decided to attempt the purification of the vitamin by means of fractional precipitation with various

TABLE II

Solubility of the Vitamin in Presence of Basic Precipitants

	Basic precipitation	Recovery of vitamin	Degree of concentration based on N	Total vitamin recovered
		per cent		*per cent*
Precipitate....................	Picric acid	40	3.5 ×	
Water layer.................	" "	30	−1 ×	
Solvent "	" "	20	−2 ×	90
Water "	Flavianic acid	20	−1 ×	
Solvent "	" "	75	2 ×	95
Precipitate....................	Rufianic acid	35	1 ×	
Water layer.................	" "	35	1 ×	
Solvent "	" "	15	−5 ×	85

organic solvents. The experiments were conducted as follows: The vitamin solution obtained by the oxidation technique was evaporated to dryness and taken up in a measured amount of absolute ethyl alcohol or glacial acetic acid. An aliquot was put into an Erlenmeyer flask, cooled to 0°, and 2.5 volumes of the ice-cold solvent were added slowly from a burette. The flask was allowed to stand at 4° overnight, the liquid decanted off, and the solvents removed, and the vitamin contents of the filtrate and precipitate determined. The results are presented in Table III. It will be noticed that in every case when glacial acetic acid was used as a solvent the recovery was not complete. The loss in the presence of acetic anhydride is particularly large (Table

TABLE III

Fractional Precipitation of the Vitamin by Organic Solvents

Experiment No.	Solvent	Precipitant	Total solids per pigeon unit	Degree of concentration based on total solids	Recovery	Total recovery
			mg.		per cent	per cent
1	Water		15		100	
2 a	C_2H_5OH	CCl_4	7	2×	95	
b	"	"			5	100
3 a	"	$CHCl_3$	11	1.5×	30	
b	"	"	35		30	60
4 a	"	$C_2H_2Cl_4$	21		20	
b	"	"	11	1.5×	65	85
5 a	"	C_2Cl_4	17		40	
b	"	"	38		20	60
6 a	"	$C_2H_4Cl_2$	5	3×	60	
b	"	"	34		25	85
7 a	"	$O{<}\!\!\begin{smallmatrix}CH_2CH_2\\CH_2CH_2\end{smallmatrix}\!\!{>}O$	5	3×	70	
b	"	"	36		30	100
8 a	"	$(CH_3)_2CO$	4	4×	70	
b	"	"	43		20	90
9 a	CH_3COOH	CH_3COOCH_3	24		40	
b	"	"	18		20	60
10 a	"	$CH_3COOC_2H_5$	11		55	
b	"	"	26		25	80
11 a	"	Iso-$C_5H_{11}OH$	28		45	
b	"	"	28		30	75
12 a	"	$\begin{smallmatrix}CH_3CO\\CH_3CO\end{smallmatrix}{>}O$	13	.	30	
b	"	"	25		10	40
13 a	"	$(C_2H_5)_2O$	26		45	
b	"	"	11		15	60
14 a	"	$n\text{-}C_4H_9OH + (C_2H_5)_2O$	33		25	
b	"	"	14		40	65
15 a	"	$O{<}\!\!\begin{smallmatrix}CH_2CH_2\\CH_2CH_2\end{smallmatrix}\!\!{>}O$	19		45	
b	"	"	19		25	70
16 a	"	$(CH_3)_2CO$	17		30	
b	"	"	16		55	85
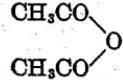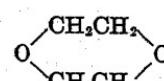
In each experiment *a* is the precipitate; *b* is the filtrate.

III, Experiment 12). No mention of the destructive action of glacial acetic acid on the antineuritic vitamin had appeared in the literature at the time these experiments were carried out. Since then, Van Veen (3) has reported that the pure vitamin hydrochloride is destroyed by acetylation.

The results, summarized in Table III, indicate that the vitamin can be concentrated by certain of the reagents employed, namely carbon tetrachloride, ethylene chloride, dioxane, and acetone. In each of the four cases mentioned, it may be noted that when the vitamin is precipitated, the organic impurities remain in solution. These data indicate that it should be possible to extend this type of fractional precipitation so as to yield eventually a highly purified vitamin solution.

While we were carrying out the fractionations described above, it seemed desirable to investigate the possibility of separating the vitamin from the organic acids obtained by the carbon tetrachloride-oxidation technique by precipitating either the vitamin or the organic acids by means of metallic salts. The following salts were tried: $Al_2(SO_4)_3$, $Al(OH)_3$, $Ba(C_2H_3O_2)_2$, $BaCO_3$, $SbCl_3$, $Ca(C_2H_3O_2)_2$, $CaCO_3$, $CaHPO_4$, $Ca_3(PO_4)_2$, $Ca(H_2PO_4)_2$, $CdCl_2$, $CoCl_2$, $CoSO_4$, $Cu(C_2H_3O_2)_2$, $CuCO_3$, Cu_2Cl_2, $CuCl_2$, $Cu(NO_3)_2$, $CuSO_4$, CuO, $FeSO_4 \cdot (NH_4)_2SO_4$, $FeSO_4$, $Pb(C_2H_3O_2)_2$, $PbCO_3 \cdot Pb(OH)_2$, $Pb_3(PO_4)_2$, Pb_3O_4, PbO, $LiCO_3$, $MgCO_3$, $HgNO_3$, $Hg(C_2H_3O_2)_2$, $HgSO_4$, HgO, $Hg(NO_3)_2$, $HgCl_2$, $Sr(C_2H_3O_2)_2$, $SrCO_3$, $SrCl_2$, $SnCl_2$, $UO_2(C_2H_3O_2)_2$, $Zn(C_2H_3O_2)_2$, $ZnCO_3$, $ZnCl_2$, ferric citrate, ferric ammonium citrate, and silver lactate. Preliminary experiments indicated that only five of these salts gave results which were worth repeating more carefully. These experiments were carried out in the following manner. To a vitamin solution obtained by the oxidation technique (6), containing about 15 per cent of total solids, a 10 per cent solution of the salt was added until no further precipitate appeared. The reaction flask was allowed to stand at 4° overnight and the precipitate centrifuged and washed with dilute reagent. After removal of the reagents both the precipitate and filtrate were tested for vitamin potency by our usual method. The results, summarized in Table IV, indicate that only mercuric nitrate and cuprous chloride give significant concentrations. It is interesting to observe that in the presence of a strong reducing agent like stannous chloride the

vitamin was almost completely destroyed.   This one experiment raises the question as to whether certain impurities in the solution have the power of protecting the vitamin against the destructive action of small amounts of reducing agents, a protection which may be overcome by larger amounts of the reagent, with consequent destruction of the vitamin.   This phenomenon seems to take place with oxidizing agents (6).

It is interesting that, in contrast to all the cupric salts tried, cuprous chloride precipitated a considerable amount of the vitamin.   The precipitating action of cuprous salts on the sulfhydryl group is well known.   The action of cuprous chloride on the

TABLE IV

Experiment No.	Precipitant	Tota lsolids per pigeon unit	Degree of concentration based on total solids	Recovery
		mg.		*per cent*
1 a	$Hg(C_2H_3O_2)_2$	16	1×	20
b	"	52	−3×	20
2 a	$Hg(NO_3)_2$	4	4×	45
b	"	13	1×	55
3 a	$SnCl_2$			*
b	"			*
4 a	Cu_2Cl_2	2.5	6×	45
b	"	20	−1.5×	55
5 a	Ag lactate	12	1×	20
b	" "	28	−2×	55

In each experiment *a* is the precipitate; *b* is the filtrate.
*Practically complete destruction.

vitamin might be due to the formation of an insoluble Cu·S·R compound.   The fact that Windaus and his associates (4) have found sulfur in the purified vitamin might be regarded as supporting this suggestion.

Confirmatory Tests for Antineuritic Potency

There have been frequent reports in the literature claiming the necessity of water-soluble factors for growth, weight maintenance, etc., in the pigeon as well as in other species; as our method of assay is primarily dependent on the maintenance of weight of the pigeon, it seemed advisable to perform confirmatory tests

for antineuritic potency on other species. As this work will be reported *in extenso* elsewhere, only a brief summary is necessary at this point.

Dog—Dogs were fed a highly purified diet as free as possible of all recognized water-soluble accessory factors. The first noticeable symptom of any deficiency was anorexia. When the vitamin concentrate was given intravenously or orally, a decided stimulus to the appetite resulted. Cowgill (12) has repeatedly pointed out that the first sign of vitamin B_1 deficiency is a loss of appetite. We believe that these positive results indicate the potency of our concentrate in this factor. Dogs in a state of paralysis were cured within a few hours by the injection of 20 to 40 pigeon units of our concentrate.[1]

Rat—Young rats were fed a diet of purified casein, starch, lard, and salts, supplemented with 0.2 pigeon unit per day of the carbon tetrachloride-oxidation concentrate, as well as small amounts of vitamin B_1-free autoclaved yeast or a commercial liver concentrate. The animals grew well and appeared normal in all respects. However, when other animals received the basal diet without the autoclaved yeast or liver supplement, they lost weight and died before developing typical skin lesions. These results might be interpreted to indicate that our concentrate, although very potent in the antineuritic factor, lacked some substance (or substances) other than the antidermatitis factor which is supplied by the crude yeast and liver preparations. Similar results have been obtained by Drs. Brand and Stucky of the New York State Psychiatric Institute and Hospital, to whom we are indebted for permission to report their findings prior to publication.

Test on Human Being—A very dramatic result was obtained in the case of a woman exhibiting the symptoms of sprue. The patient, a native of Porto Rico, had been gradually losing weight over a period of three years, decreasing from over 200 pounds to about 90 pounds. She had the typical symptoms of extreme sprue—anemia, anorexia, muscular weakness, diarrhea, and inability to retain nourishment given by mouth. She was given an intensive treatment for her anemia by the injection of large quantities of liver concentrate; repeated attempts were

[1] We are indebted to Dr. Ethel Burack for making these tests.

made to correct her anorexia by the ingestion of Bemax, a wheat germ product, and similar preparations known to contain antineuritic vitamin B. In spite of these therapeutic measures, her condition became progressively worse. Apparently the vitamin supplied by these preparations was not absorbed from the alimentary tract. As a last resort, 80 pigeon units of the antineuritic concentrate previously described by us (6) were injected intramuscularly. Although the injection was quite painful, the patient soon recovered from the ill effects and within a few hours became interested in food and for the first time in several weeks was able to retain what she had eaten. Since the injection, she has been able and willing to eat and her treatment for sprue is leading to a rapid cure.

The pain caused by the injection of this particular concentrate is quite a distressing feature and prohibits its clinical use except in such extreme cases as the one just mentioned. However, we have obtained some preliminary results indicating that under certain conditions the vitamin is soluble in ether and other common organic solvents, whereas the acids of carbohydrate origin (?) are not. This procedure leads to a concentration calculated on the basis of the nitrogen of about 300 to 500 times. By this technique about 75 per cent of the initial vitamin potency is recovered.

SUMMARY AND CONCLUSIONS

Various modifications and simplifications of the carbon tetrachloride-oxidation technique (6) are described. These modifications result in a highly potent, salt-free vitamin B (B_1) concentrate which can be quickly and easily prepared in the average chemical laboratory.

Treatment of the vitamin solution thus obtained with nitrous acid does not reduce the total amount of nitrogen present and above certain concentrations results in a destruction of the antineuritic substance.

Attempts to purify the vitamin through the picrate, flavianate, or rufianate by ether-butyl alcohol mixtures are described. Some of the results obtained by this technique were suggestive.

Fractional precipitation of the vitamin from non-aqueous solutions by various organic solvents yielded favorable results.

Destruction of the vitamin by strong acetic acid and especially by acetic anhydride was observed.

Approximately 50 inorganic salts were used in an attempt to purify the vitamin obtained by the oxidation procedure. Under the conditions employed only cuprous chloride and mercuric nitrate gave worth while results. Stannous chloride caused an extensive destruction of the vitamin.

The potency of our concentrate has been tested with favorable results on pigeons, rats, and dogs.

A brief report is made of the effect of the parenteral administration of one of our concentrates to a human being with sprue. Although a dramatic cure resulted, at present we strongly advise against parenteral use of this concentrate in humans except *in extremis.*

The chemical procedures were carried out at the Psychiatric Institute; the biological assays at New Haven.

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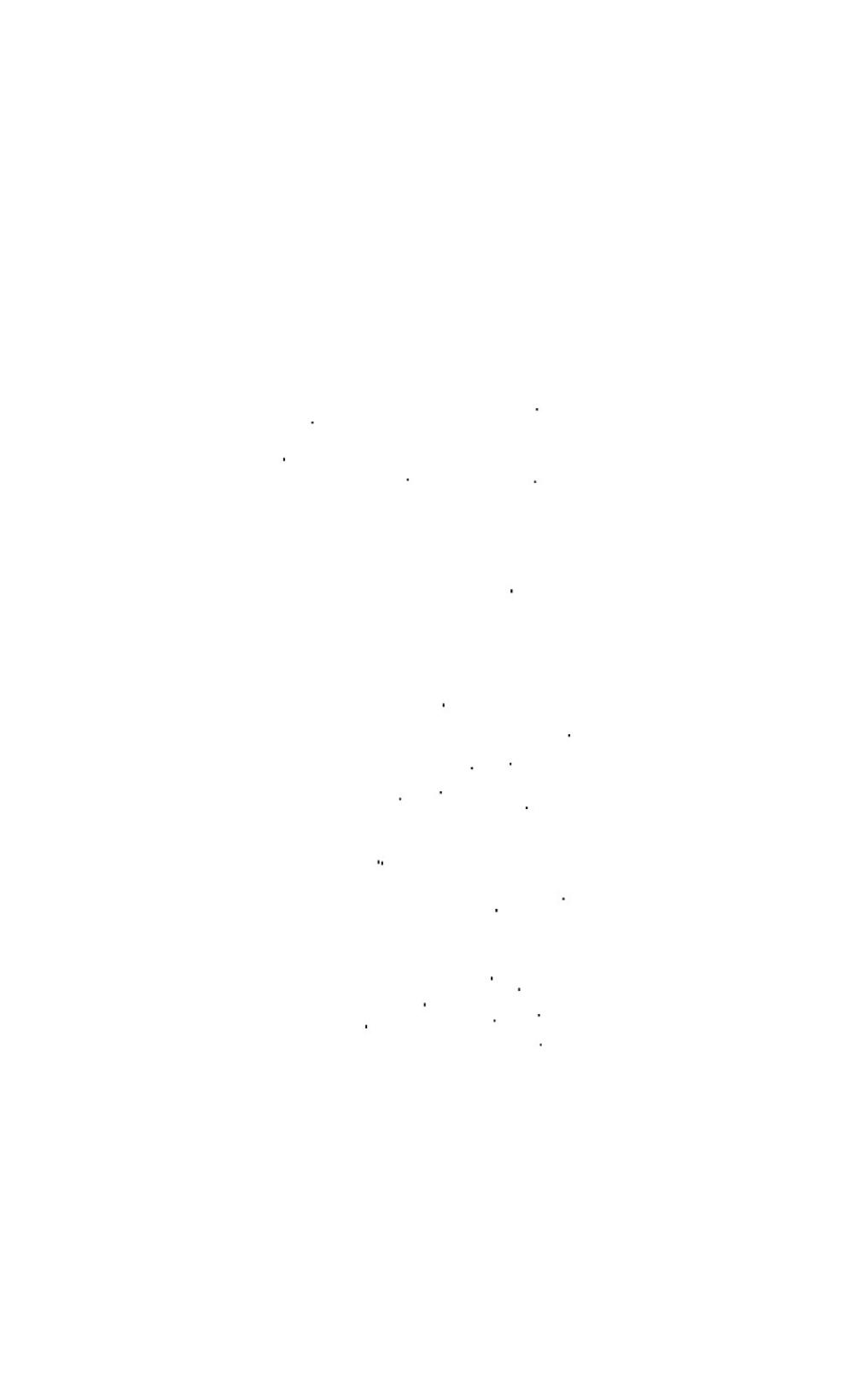
ACID-BASE BALANCE IN SWEAT

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The reaction, the osmotic pressure, and the volume relation-ships of the body fluids are closely dependent on the properly regulated acid-base equilibrium. We have attempted to work out some of the regulatory mechanisms for the preservation of acid-base equilibrium during the process of heavy sweating in ways analogous to those employed in the many similar studies of blood and urine. The changes to be recorded take place within a comparatively short space of time, so that we can study the factors for regulating acid-base balance without the interfering effects of the intake of food and the loss of catabolites in the urine and feces (the subjects void minimal amounts of urine); also the compensatory mechanisms whereby the loss of fixed base is reduced to a minimum at a time when the body fluids are undergoing marked changes become apparent.

It is extremely difficult by ordinary means to cause a substantial elevation of body temperature. We have therefore made use of the so called radiotherm. This machine produces a rapidly varying electrical field of a frequency of about 10 million cycles between the plates of a condenser. The body of the subject rests between the plates, but not in contact with them. The body is a poor dielectric and hence at such frequencies heat is produced. This heating is developed throughout the body, and there is no great temperature gradient at any point. In order to deliver heat at the same rate with ordinary methods of heating, a very great temperature gradient from the surface inward would be required, resulting necessarily in skin temperatures dangerous to the subject.

The heating of the patients requires approximately 1 hour, as can be seen from the temperature curve (Fig. 1). After the

temperature has been elevated, the patients are removed to a bed which is surrounded by heating lamps, and the temperature maintained at a high level for approximately 4 hours. The sweat is collected at intervals on cotton from the entire surface of the body. Since the subjects used in this investigation suffered from pathological conditions as varied as paresis, tabes, acute gonorrhea, arthritis, scleroderma, psoriasis, etc., we are justified in using the
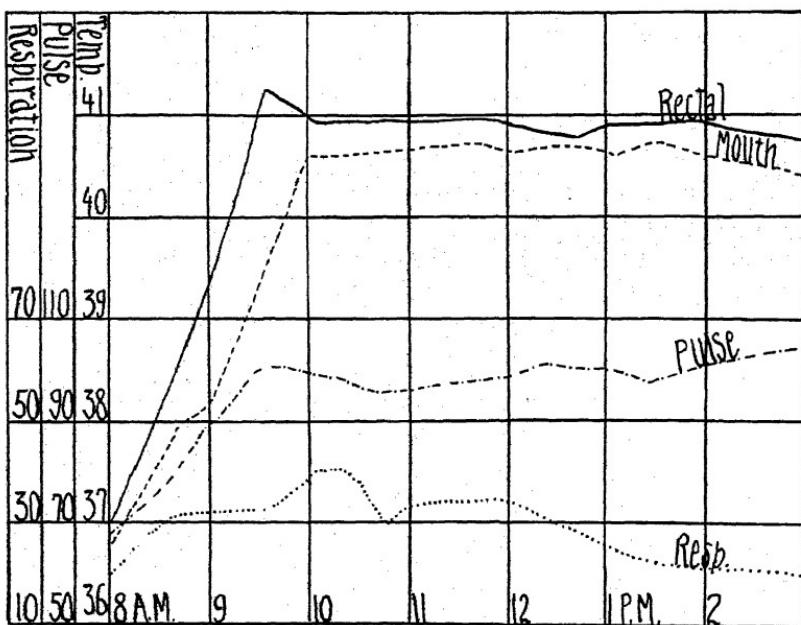
Fig. 1. Temperature, pulse, and respiration of patient in radiotherm

results obtained as fairly independent of the specific pathological condition of the patient.

We were able to attain temperatures as high as 42°, but in the average patient it was raised to about 41°. There was an enormous loss of body fluid through sweating; in fact, this almost equaled the entire blood volume. It is known that miners during work can lose as much as 3000 cc. of body fluid per hour. Dill and coworkers (1) found that athletes lost as much as 2.1 kilos of fluid during a 2 hour race.

During exertion and at high temperatures, the lack of balance

between fixed base and acid catabolites is augmented, and some regulatory mechanism is essential if the fixed base depots of the body are not to be drained. The base economy factor of the skin will depend on its ability to produce a fluid of lower pH than the blood plasma, which acts as the carrier of the catabolic products. There has been a long controversy as to the reaction of the sweat, which has only been satisfactorily settled by the introduction of the electrometric method of pH determination. As early as 1833 Thenard (2) reports the sweat as definitely acid. It is known that athletes complain of "stinging sweat." It has been shown by Marchionini (3) that the divergency of the results obtained was due principally to the admixture of much apocrine sweat, which is distinctly alkaline. Schiefferdecker (4) pointed out that this apocrine sweat was secreted by special glands in the axilla and genitoperineal region, and is very rich in odoriferous substances and cell content. In heavy sweating the proportion of this latter type of sweat becomes very small, and we get results that more exactly measure the true pH of the exocrine sweat due to exertion in heat. In our cases the pH of the sweat as determined by the hydrogen electrode method varies between 4 and 4.5.

At a range of pH as low as this, several important changes come into play. The acid substances are carried bound in the plasma for excretion and consist of carbonates, phosphates, sulfates, chlorides, the salts of organic acids, and proteinates. All of these appear in the sweat except carbonates and proteinates. The skin is impermeable to the protein, while at the low pH carbonates as such are practically non-existent. Thus, a wasteful expenditure of base on a catabolite which can be excreted practically base-free through the lungs is avoided. A similar mechanism has been shown by Gamble (5) to operate in acid urines. Thus the carbonates and protein do not enter the sweat but remain in the plasma and exert their function in stabilizing the reaction of the blood.

The presence in the sweat of an acid which would be ionized to only a relatively low degree would allow of the excretion of a large amount of acid catabolites, without undue strain on the fixed base of the body. Such an acid we found in lactic acid, which, at the pH of sweat, is about 50 per cent ionized; also, in addition

to its base-saving properties, it can act as a powerful buffer. It is present in the sweat in a concentration of about 250 to 350 mg. per 100 cc. The chloride ion content of the sweat is about the same quantity. If we hold in mind that the chloride ion content of the plasma is about 0.35 per cent, and the lactic acid 0.015 per cent, it is evident that the Cl content of the plasma is about equal to that of the sweat, while the lactic acid is 20 times higher in the sweat than in the plasma. Hence, the skin functions simply as an ultrafilter with regard to chloride, while it can concentrate the lactic acid to high amounts. It is significant that just those constituents which are excreted by the kidney at practically the same concentration as in the plasma are also excreted in the same ratio in the sweat; namely, Cl^-, Na^+, and Ca^{++}.

It is peculiar that the presence of lactic acid in sweat is scarcely mentioned in the literature. Liebermann (6), in a text-book in 1880, states that in patients suffering from purpuric fever, lactic acid is found in the sweat. Schenck (7) found in the sweat of subjects after light exertion 200 to 300 mg. of lactic acid. Snapper and Grünbaum (8) found quantities of lactic acid of over 1 gm. in the shirts of football players after the Olympic Games. When it is considered that the shirt covered only a portion of the body, it can be seen that the actual quantity of lactic acid excreted must have been higher.

The blood lactic acid content is known to rise after severe exertion. Barr, Himwich, and Green (9) report its rise to 100 to 120 cc. Loebel, Barr, Tolstoi, and Himwich (10) found the lactacidemia in dogs after strychnine convulsions as high as 150 cc. The results as found in animal experiments can hardly be employed for comparative purposes because of the difference in the sweating mechanisms between animals and man. In our patients the lactic acid in the blood usually rose to between 60 and 70 mg. per 100 cc. Observations of the alveolar air after exercise by Haldane and Quastel (11) indicate that only a small proportion of the total lactic acid formed in the muscles finds its way into the blood. Thus the alveolar CO_2 pressure was only lowered by 8.6 mm. of mercury, which would correspond to neutralization of bicarbonate by about 2.8 gm. of lactic acid in the blood of the entire body. If this is so, the authors conclude that the muscle must retain most of its lactic acid. We have found a

rise of lactic acid in the blood of an approximately similar magnitude in our subjects but could account for a larger proportion of the lactic acid which was excreted in the sweat. It is of some interest that in experiments, such as in calculating the oxygen deficiency after violent exertion, more consideration should be given to the influence of the sweat production on the concentrations of lactic acid, because 6 or 7 gm. of lactic acid can be accounted for in this fashion.

For the purposes of better illustrating the acid-base balance, the following constituents were determined in the blood and

TABLE I

Acid-Base Composition of Blood Plasma and Sweat

Water lost	Fluid	Hrs.	Na	K	Ca	Cl	Lactic acid	pH
cc.			*cc.* 0.1 N	*cc.* 0.1 N	*cc.* 0.1 N	*cc.* 0.1 N	*cc.* 0.1 N	
4020	Blood	0	142	5.1	5.1	103	1.3	7.45
		3	143	5.0	5.1	102	5.1	7.32
	Sweat	1	82	5.1	5.7	85	25.1	4.10
		3	83	4.9	5.6	83	25.2	4.05
3350	Blood	0	141	5.1	4.5	103	2.2	7.44
(Entirely through skin;		3	142	5.0	4.1	101	6.1	7.31
allowance for loss by	Sweat	1	75	5.7	5.6	74	24.0	4.18
respiration)		3	80	5.3	5.5	72	23.0	4.12
1542	Blood	0	142	5.1	5.1	103	1.0	7.42
(Fluids restricted)		3	145	5.9	5.1	106	4.2	7.38
	Sweat	1	81	10.05	5.3	69	34.0	4.30
		3	82	9.83	5.5	64	30.0	4.25

sweat: pH, sodium, potassium, chloride, phosphate, carbonate, lactic acid. These are given in Table I in acid-base values as 0.1 N. The typical acid-base balance in the sweat is illustrated by Fig. 2.

If we titrate a solution of weak lactic acid with its equivalent of sodium hydroxide at 37.5°, we find that at the half titration point the pH of the solution is 3.94. Using the Hasselbalch form of the Henderson equation, where Ba is the concentration of acid salt and Ha of undissociated acid, we have

$$pH = pK + \log \frac{Ba}{Ha}$$

and hence can find the dissociation constant of lactic acid at this temperature, which was found to be 1.23×10^{-4}. Using the equation form

$$pH = pK + \log_1 \frac{\text{--}}{\text{--}}$$

we find that at the pH of sweat, lactic acid is approximately half dissociated. Hence the lactic acid can be excreted to a large
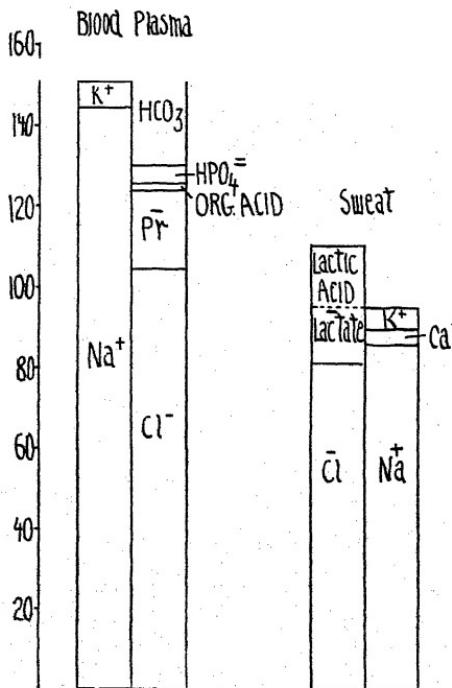
FIG. 2. Acid-base balance expressed in cc. of 0.1 N HCl after loss of 3500 cc. of body fluid due to temperature elevation to 41.5°.

extent unionized, resulting in a sparing of 15 units of 0.1 N base per 100 cc. When we consider that we are dealing with approximately 3000 cc. of fluid, the absolute sparing of base is large.

The proportion of approximately one-half lactic acid in the presence of one-half of its alkali salt can act as a powerful buffer. Van Slyke (12) has shown that the buffer power of a solution can be accurately measured thus. All monovalent buffers acting

within the range of validity of Henderson's equation with pK between 4 and 10 have the same maximum molecular buffer value, *viz.* $\beta m = 0.575$, which they exert when $K = [H^+]$ or pH = pK'. At this pH, (Ba) = (Ha) = $\dfrac{C}{2}$; that is, half the buffer acid is free, half in the form of the alkali salt. If the point of maximum
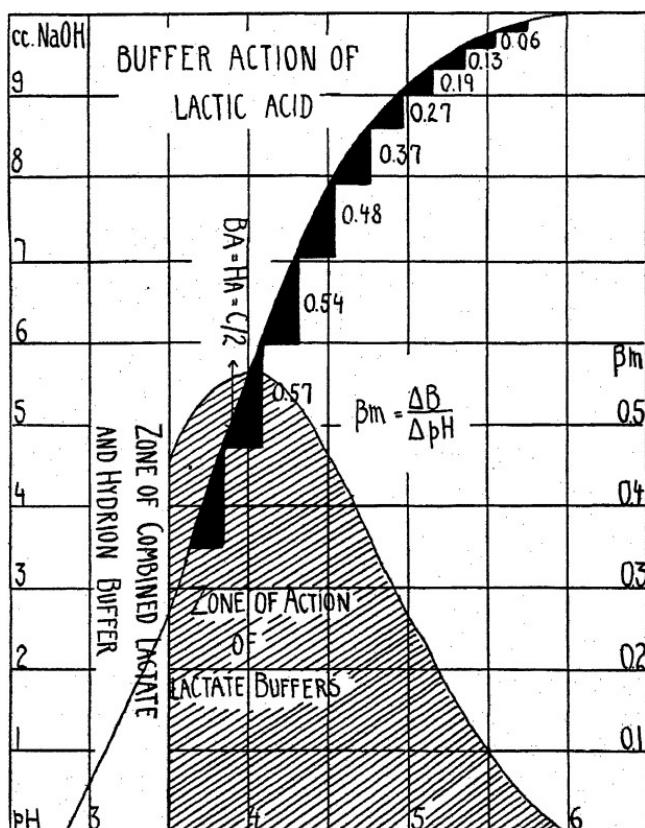
FIG. 3. Titration of lactic acid with equivalent NaOH

slope be determined in a buffer curve, with increments of added alkali as ordinates and pH values as abscissæ, one may calculate the molecular concentration of the buffer by dividing the maximum slope of the titration curve by 0.575. In Fig. 3 the titration curve of 0.02 N lactic acid with equivalent NaOH is given. It can be seen that the maximum buffer action occurs at approximately

pH 4, and the tangents of the curve at intervals of 0.2 pH are given. Below pH of about 3.5 the buffer power cannot be so simply measured because the buffer effects of the lactate and hydrion overlap. As this is of no special importance for our problem because it is outside the physiological range, we have not taken it into consideration. The buffer value of sweat is approximately that of a 0.02 N solution of a buffer acid with $K = 10^{-4}$.

The actual stimulus for the secretion of sweat is in dispute. It seems possible that the lactic acid formed by heat and exercise may be the actual substance initiating this process and that the lowered pH is necessary for proper function of the glands. The buffer action would then keep the optimum pH. Some mention should be made in this connection of the phylogenetic homology between the mammary glands and the sweat glands.

The methods used in this work for the determination of the various constituents are as follows: sodium (Kramer and Gittleman), potassium (Kramer and Tisdall), lactic acid (Clausen), phosphorus (Briggs), calcium (Kramer and Tisdall), total base (Fiske), chloride (Van Slyke), carbon dioxide (Van Slyke). These methods may be found in detail with critical comments in Peters and Van Slyke (13).

SUMMARY

A study was made of the acid-base equilibrium in patients whose temperatures were raised to approximately 41° and whose loss of fluid practically equaled the entire blood volume. The base economy factor of the skin was found in the ability to excrete the sweat at a much lower pH than the blood plasma. Also the excretion of lactic acid and lactates resulted in the sparing of fixed base because of the proportion of lactic acid passing out unionized. The presence of lactic acid and lactates acted as a buffer to prevent the lowering of the pH to below 4, with consequent damage to the skin. It is possible that the lactic acid produced at high temperatures and by exercise may be the actual stimulus for initiating the function of the sweat glands.

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THE PROTECTION OF INSULIN BY ANTIPROTEASES, AND ITS ABSORPTION FROM THE INTESTINE

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Representative attempts at enteral administration of insulin have been directed to three main objectives: (1) inhibition of protease activity, especially tryptic activity; (2) increase in permeability of the intestinal wall; (3) preparation of insulin derivatives or substitutes which are not affected by digestive enzymes. The abundant literature may be fairly summarized as having demonstrated results qualitatively successful but quantitatively insufficient to establish the oral route as an effective or practicable mode of insulin therapy. While this record and the inherent physical difficulties imposed by the indicated protein structure[1] of insulin discourage further efforts to improve the efficacy of oral administration of the hormone, the conditions do not appear to us to be sufficiently conclusive to stamp such undertakings with a prediction of absolute futility.

In view of the relatively considerable success obtained by Murlin and Hawley (2) with mixtures of blood serum[2] and insulin which were given by stomach tube to depancreatized dogs, we have prepared and tested more concentrated and potent antiprotease mixtures. Our results fall into two categories: (1) the protection of insulin from proteolytic inactivation *in vitro*; (2) the absorption from the alimentary canal of insulin protected by antiproteases.

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[1] During the course of our work Sjögren and Svedberg (1) reported the molecular weight of crystalline insulin to be 35,000.

[2] Blood serum contains antiproteases, notably antitrypsin. Since tryptic activity in the intestine of the depancreatized dog would appear to be essentially nil any beneficial effects of blood serum would be attributed to inhibition of other proteases.

Preparation of Antitrypsin

Highly concentrated preparations of antitrypsin, which exhibited, also, marked antipeptic properties and perhaps contained other antiproteases, were obtained from the roundworm of swine, *Ascaris lumbricoides*, a modification of Hamill's directions (3) being followed. The worms were frozen thoroughly by means of carbon dioxide snow and ground coarsely in a small meat chopper. After the ground particles had thawed slightly they were passed through a finer attachment in the same mill. The well macerated material was suspended in 1.5 volumes of water with a small amount of chloroform as preservative, and the mixture was agitated overnight by a motor-driven stirrer. The suspended material was then removed by centrifugation and discarded. To the liquid was added 95 per cent ethanol in amount sufficient to produce a final alcohol concentration of 66 per cent. After standing 5 to 6 hours the heavy precipitate was filtered off and discarded. The alcohol concentration of the filtrate was increased to 85 per cent and 1 N sulfuric acid added to produce a final acidity of 0.001 N. After standing about 10 hours the white precipitate adhering to the sides and bottom of the glass container, and representing concentrated, though impure, antitrypsin, was separated readily from the liquid by siphoning the major portion of the latter and centrifuging the final part. The most evident impurity in the crude antitrypsin is a protease, active in alkaline solution and not inhibited by antitrypsin. Purification was accomplished by dissolving the highly soluble precipitate in water and subsequently separating antitrypsin from impurities by fractional precipitation with ethanol. In this fractionation addition of a small amount of solid sodium chloride facilitated flocculation. Each successive precipitate was separated from the mother liquor by centrifugation and dissolved in the minimum quantity of water. Both the proteolytic and antitryptic properties of each fraction were tested qualitatively. The protease content was followed by observing its digestive action, at pH 8, 37.5°, upon blood fibrin stained with Congo red, the rate and extent of proteolysis being estimated roughly from the liberation of dye into the solution. Similarly, the antitryptic potency of the respective fractions was determined by the protection afforded stained blood fibrin against the digestive action of pancreatin. For comparative purposes a tentative

unit of antitrypsin was adopted as the smallest quantity which would prevent for 6 hours, at 37.5°, the digestion of 0.10 gm. of Congo red-stained blood fibrin (10 mesh) by 10.0 cc. of 1.0 per cent filtered pancreatin (U.S.P.), in a total volume of 12.5 cc., and at pH 8 (0.5 cc. of 5 per cent $NaHCO_3$). Stock antitrypsin solutions were diluted so that 1 cc. contained 5 units. The nitrogen content of 5 units was approximately 1 mg.

Although the fractions precipitated at alcohol concentrations below 70 per cent contained appreciable quantities of antitrypsin, they were discarded because of their high protease content. The proteolytic activity of Fraction C, obtained at alcohol concentrations of 70 to 80 per cent, was very low, while the antitryptic activity was maximal. Fractions precipitated at still higher concentrations of alcohol contained negligible amounts of protease and antitrypsin. Only Fraction C was employed in experiments with insulin. In water solution, with no preservative added, this fraction retained a large portion of its original potency for 3 months in a refrigerator at 4°. Tests of its activity beyond this period were not made.

In Vitro Protection of Insulin by Antitrypsin

Methods and Procedure

After qualitative experiments had shown that isoelectric insulin could be recovered following its incubation for 6 hours with strong pancreatin solutions, at pH 8, 37.5°, in the presence of antitrypsin but not in its absence, a semiquantitative procedure was adopted for determining the degree of protection afforded by the antitrypsin. The course of insulin digestion under these conditions was followed at selected intervals by measuring the non-protein nitrogen values of the digests and by assay of the digests in rabbits.

Non-protein nitrogen values were determined by a micro-Kjeldahl distillation-Nesslerization procedure on the Somogyi (4) zinc hydroxide filtrate. While intermediate products of digestion by the pancreatin may have been precipitated by zinc hydroxide, final products, as amino acids and ammonia, remained in the filtrate to serve as a satisfactory index of digestion.

Blood sugar was determined by the Shaffer-Hartmann method, with 0.2 cc. of blood, the Somogyi (5) modification of the reagent

being used. Blood was drawn from the jugular vein in dogs and the marginal ear vein in rabbits, without the use of xylene. For urine sugar the Shaffer-Hartmann technique was used after the preliminary mercuric sulfate treatment of West, Scharles, and Peterson (6).

Our biological assay of insulin followed the procedure (7) of injecting the insulin mixtures subcutaneously into approximately 2 kilo, male, albino rabbits, previously fasted for 24 hours, and determining the blood sugar values at 1.5, 3.0, and 5.0 hour intervals. Concentrated stock insulin solutions,[3] containing according to the manufacturer's assay 340 or 520 clinical units per 1.0 cc., were diluted as required. Preliminary assays of these solutions upon a limited number of rabbits checked satisfactorily with the supposed value.

<div align="center">EXPERIMENTAL</div>

Witzemann and Livshis (8) found that in slightly alkaline solutions at room temperature trypsin inactivated insulin with an increase of 50 per cent in the formol titration in 42 hours. Epstein and Rosenthal (9) have published results indicating that trypsin forms with insulin an inactive complex from which active insulin may be recovered. A detailed study of the phenomenon reported by Epstein was made by Scott (10), who believes that in strongly acid solutions trypsin inactivates insulin by forming a combination similar to the adsorption complexes formed by charcoal with various substances; active insulin may be liberated from this combination. At pH values of 7.5 to 9.0, however, insulin is irrevocably inactivated by trypsin. Harteneck and Schuler (11) state that in slightly alkaline solution the reaction between trypsin and insulin conforms in all respects to the laws governing enzymatic digestion and is irreversible. As may be seen from our results in Table I, a rapid increase occurs in the non-protein nitrogen of pancreatin-insulin digests when antitrypsin is not present, indicating an irreversible destruction of the insulin. It is apparent, also (Solution 4, Table I), that antitrypsin effectively inhibits at least the final steps in the hydrolysis of insulin by pancreatin.

The effectiveness of antitrypsin in preventing destructive changes

[3] The large quantities of insulin used in our experiments were generously provided by Eli Lilly and Company.

in the physiological activity of insulin which may not be reflected in non-protein nitrogen values should be determinable by biological assay.   Having ascertained that neither pancreatin nor pancreatin plus antitrypsin affects the blood sugar level in rabbits when given in the concentrations employed in the insulin digests, we carried out upon rabbits a large number of assays, typical examples

TABLE I

Antitryptic Inhibition of Insulin Digestion (March 6, 1931)

5 cc. of each solution were diluted with 10 cc. of water and precipitated by 1 cc. each of 19.5 per cent $ZnSO_4$ and 1 N NaOH.   The nitrogen in 8 cc. of filtrate, corresponding to 2.35 cc. of original solution (18.85 units of insulin), was measured.   The pancreatin was U.S.P. (Coleman and Bell).   Incubation temperature was 37.5°.

	Solution 1	Solution 2	Solution 3	Solution 4
	cc.	*cc.*	*cc.*	*cc.*
Filtered pancreatin (Sample 1), 1 per cent..............................	20.0	20.0	20.0	20.0
$NaHCO_3$, 5 per cent..................	1.0	1.0	1.0	1.0
Insulin (200 units in 3.0 cc.)..........	0.0	0.0	3.0	3.0
Antitrypsin (Sample 1) (5 units in 1.0 cc.).................................	0.0	1.0	0.0	1.0
Water...............................	4.0	3.0	1.0	0.0

Non-protein N in 12.5 cc. (100 units insulin) original solution

	mg.	*mg.*	*mg.*	*mg.*
Immediately after mixing.............	0.341	Lost	0.490	0.420
After 2.5 hrs. incubation..............	0.352	"	0.674	0.466
" 4.5 " "	0.390	0.455	0.741	0.490

Increase in non-protein N

	per cent	*per cent*	*per cent*	*per cent*
After 2.5 hrs. incubation..............	3.2		37.6	9.5
" 4.5 " "	14.4		51.2	16.7

of which are presented in Table II.   Three other series of experiments in which we injected digests in amounts corresponding originally to 12, 8, and 6 units of insulin, respectively, gave results qualitatively similar.   All conditions and quantities, except insulin concentrations, were identical with those recorded in Table II.   In each instance, in the absence of antitrypsin, the activity

of the insulin was destroyed completely during the first incubation period. However, when protected by antitrypsin, injection of digests incubated for 2.5 and 4.5 hours reduced the blood sugar to

TABLE II

Bioassay of Pancreatin-Insulin Digests, with and without Antitrypsin (May 15, 1931)

The rabbits were fasted for 20 hours; incubation temperature, 37.5°; pH of each solution, 8.

	Hrs. after injection	Solution 1* 2.0 cc. (6 units insulin) injected			Solution 2* 1.0 cc. (3 units insulin) injected			
		Rabbit No.	Weight	Blood sugar	Rabbit No.	Weight	Blood sugar	Insulin found
			kg.	*mg. per cent*		*kg.*	*mg. per cent*	*units*
Injections immediately after mixing	Control	1	2.35	114	3	2.20	101	
	1.5			112			52	
	3.0			109			52	
	5.0		·	103			54	2.73
Injections after 2.5 hrs. incubation	Control	4	2.40	114	6	2.30	114	
	1.5			112			59	
	3.0			114			57	
	5.0			103			55	2.84
Injections after 4.8 hrs. incubation	Control	7	2.20	112	9	2.45	114	
	1.5			109			49	
	3.0			92			52	
	5.0			100			70	3.04

* The composition of the solutions was as follows:

	Solution 1	Solution 2
	cc.	*cc.*
Filtered pancreatin (Sample 2), 1 per cent.	40.0	40.0
NaHCO$_3$, 5 per cent	2.0	2.0
Insulin (150 units in 0.44 cc.)	0.44	0.44
Antitrypsin (Sample 2) (5 units in 1.0 cc.).	0.0	2.0
Water	7.56	5.56

or below 45 mg. per cent in every case, resulting in hypoglycemic convulsions in 50 per cent of the rabbits. These experiments confirm a remarkably effective protection of insulin by antitrypsin.

In the experiments of Table II smaller quantities of insulin were employed with the view of avoiding convulsions in the rabbits, thus permitting a more precise estimation of the limits of protection which may be afforded by antitrypsin. The calculated protective value of the antitrypsin was 91 per cent when the mixture of insulin, pancreatin, and antitrypsin was injected immediately, 96 per cent after incubating the mixture for 2.5 hours at 37.5°, and 101 per cent after 4.8 hours incubation. Thus, after approximately 5 hours incubation of insulin in 0.8 per cent pancreatin under conditions optimal for digestion except for the presence of antitrypsin, at least 90 per cent of the original insulin activity was preserved. Without antitrypsin, under otherwise duplicate conditions, insulin was inactivated so promptly that injection of the original equivalent of 6 units immediately after mixing was entirely without effect upon the blood sugar level (Rabbit 1).

We would not insist from our data that complete destruction of unprotected insulin by pancreatin may be effected within the brief interval (never more than 2 minutes) elapsing between mixing and the first injection. Digestion may continue at the site of subcutaneous injection before appreciable absorption has occurred. In all cases, however, it appears that, without protection by antitrypsin, insulin activity was completely destroyed by pancreatin long before the end of the first incubation period. No effort was made to determine the maximal incubation time during which antitrypsin might protect insulin, since we believed that a 5 hour period should cover all possibility of insulin absorption from the intestine after oral administration.

The ease with which insulin activity may be determined, the probability that this activity is dependent upon the integrity of a protein structure, and the minute quantities[4] which suffice as substrate, combine to make of insulin a unique indicator of anti-enzyme effectiveness.

Alimentary Administration of Insulin

Having demonstrated an effective *in vitro* protection of insulin against pancreatic digestion, we undertook to extend this protection to insulin administered by the alimentary route. In prelimi-

[4] The insulin used in our experiments contained 0.0115 mg. of nitrogen per unit.

nary experiments, with blood sugar values as the criterion of insulin absorption, mixtures of insulin with antitrypsin were injected directly into the lumen of the duodenum of etherized, amytalized, or decerebrate rabbits. Complicating effects of anesthetic and frequent convulsive muscular movements, however, convinced us that valid results could be obtained only upon the unanesthetized animal. Accordingly, in subsequent experiments dogs with duodenal hernias[5] were employed. A portion of the duodenum approximately 2 inches long and opposite the head of the pancreas was brought into an incision through the abdominal wall slightly lateral to the mid-line and sewed into position. The exposure was covered with vaselined gauze for the 1st week, after which all bandages were removed. When the animal was depancreatized at the same operation the bandages were retained for 2 weeks or longer, since the wounds healed more slowly and the animals were disposed to bite into the intestine. With such a preparation it is possible to inject directly into the duodenum without pain to the animal. Skin grows rapidly over the exposed gut and has completely covered it after 6 to 8 weeks in the normal dog. However, for several months the skin is not sufficiently thick to interfere with the surface landmarks of the original exposure. A marked diarrhea follows the operation for the first 3 or 4 days, after which the condition may be controlled by including large amounts of bone ash in the diet.

Duodenal Administration of Insulin to Dog with Intact Pancreas

A duodenal hernia was instituted in Dog 1, female, weighing 10.7 kilos, on July 3, 1931. Recovery was rapid. The diet consisted of dog biscuit, cooked beef heart, and bone ash. At 10.00 a.m., July 11, 41 hours after feeding, a mixture of 170 clinical units of insulin, 11 units of antitrypsin, and 0.5 cc. of 5 per cent sodium bicarbonate,[6] in a total volume of 5 cc., was injected into the duodenum. Blood samples from the jugular vein at 30, 70, 125, 190, and 260 minutes after injection showed no significant change

[5] These operations as well as the pancreatectomies were performed by Dr. Morton J. Tendler of the Division of Surgery.

[6] The white precipitate which forms when insulin and antitrypsin solutions are mixed is dissolved upon addition of small amounts of sodium bicarbonate or hydrochloric acid.

in sugar value from the control level 5 minutes before injection. On July 15, after a 40 hour fast, the experiment was repeated by doubling the quantities of insulin and antitrypsin injected without effect upon the blood sugar level.

To check the activity of the antitrypsin preparation and the responsiveness of the dog to insulin when administered subcutaneously, a mixture of 40 cc. of 1 per cent pancreatin, 2 cc. of 5 per cent sodium bicarbonate, 10 units of antitrypsin, and 150 units of insulin, in a total volume of 50 cc., at pH 8, was incubated for 2.5 hours at 37.5°. Of this digest, 10 cc., equivalent to 30 units of insulin, were injected subcutaneously into the dog, which had fasted for 42 hours. From a control level of 85 mg. per cent, the blood sugar fell within 1 hour to 45 mg. per cent and remained approximately at this value for 4 hours, when the dog was fed to avoid impending convulsions. Repetition of the experiment, with omission of antitrypsin from the insulin-pancreatin digest, was without effect upon the blood sugar.

Thus, with an active preparation of antitrypsin, and with a dog reacting normally to insulin, we failed to obtain any evidence of intestinal absorption of insulin. Explanation of this failure presents at least two interpretations: (1) under the conditions of our experiment insulin did not pass through the walls of the intestine; (2) insulin was absorbed through the intestinal wall, but in such small amounts and at so slow a rate that compensatory mechanisms in pancreas, liver, or other tissues operated to maintain a normal carbohydrate equilibrium. In support of the second alternative we may suggest that since the pancreas responds to carbohydrate administration by an increased production of insulin, its reaction to insulin administration might be expected to be a diminished secretion. It appeared desirable to test this hypothesis upon depancreatized animals.

Alimentary Administration of Insulin to the Depancreatized Dog

In Table III we have selected a few typical data of various experiments in the administration of insulin by alimentary route to a fasting, depancreatized[7] dog with duodenal hernia. The observations made upon this dog covered three experimental periods of 71, 126, and 177 hours, respectively, spaced between

[7] Autopsy confirmed complete removal of the pancreas.

TABLE III

Alimentary Administration of Insulin with and without Antiprotease Protection to Depancreatized Dog

Dog 2, female; depancreatized and duodenal hernia instituted, November 10, 1931; weight, December 18, 1931, 15.4 kilos; January 11, 1932, 14.5 kilos.

Date	Fasted	Time of collection	Urine					Blood sugar
			Volume per hr.	Acetone	Glucose per hr.	Total N per hr.	D:N	
1931	hrs.		cc.		gm.	gm.		mg. per cent
Dec. 22	96	8.00– 10.00 a.m.	41.0	+	1.50	0.41	3.66	323 (9.50 a.m.)
		10.15 " insulin*						
	98	12.00 m.	50.0	+	1.26	0.39	3.23	327 (1.00 p.m.)
	100	2.00 p.m.	36.0	+	1.17	0.35	3.34	
	102	4.00 "	51.0	+	1.19	0.36	3.30	
	104	6.00 "	36.0	+	1.26	0.34	3.70	330 (6.00 p.m.)
	106	8.00 "	33.0	+	1.11	0.33	3.36	
Dec. 23	118	8.00 a.m.	22.5	+	1.15	0.30	3.83	
Total for 24 hrs..........			764		28.79	7.96	3.61	
1932								
Jan. 14	71	7.30– 9.30 a.m.	26.0	tr.	1.75	0.30	5.85	
	73	11.30 "	26.0	"	1.00	0.27	3.70	335 (11.20 a.m.)
		11.40 " insulin †						
	75	1.30 p.m.	19.0	tr.	0.58	0.22	2.64	330 (1.00 p.m.)
	77	3.30 "	11.0	"	0.33	0.18	1.83	318 (2.00 ")
	79	5.30 "	12.0	"	0.31	0.23	1.35	333 (3.30 ")
	81	7.30 "	33.5	"	0.73	0.27	2.71	
Jan. 15	93	7.30 a.m.	12.5	+	0.55	0.20	2.77	
Jan. 16	119	7.30– 9.30 a.m.	27.0	+	1.33	0.41	3.25	
	121	11.30 "	16.0	+	0.94	0.38	2.47	348 (11.20 a.m.)
		11.40 " insulin‡						

+ , positive for acetone; tr., trace.

* The dog received by stomach tube 1000 units of insulin in 100 cc. of solution, temperature 2°.

† The dog received by stomach tube 1000 units of insulin with 75 units of antitrypsin in 100 cc. of solution, temperature 2°.

‡ 1000 units of insulin with 75 units of antitrypsin in 20 cc. of solution were injected into the lumen of the duodenum.

TABLE III—*Concluded*

Date	Fasted	Urine						Blood sugar
		Time of collection	Volume per hr.	Acetone	Glucose per hr.	Total N per hr.	D:N	
1932	*hrs.*		*cc.*		*gm.*	*gm.*		*mg. per cent*
Jan. 16—	123	1.30 p.m.	14.0	+	0.59	0.40	1.47	327 (1.00 p.m.)
Con-	125	3.30 "	9.0	+	0.03	0.33	0.09	330 (2.00 ")
tinued	127	5.30 "	6.0	+	0.07	0.32	0.22	320 (3.30 ")
	129	7.30 "	20.0	+	0.13	0.36	0.36	
Jan. 17	141	7.30 a.m.	27.0	+	1.58	0.34	4.65	
Total for 24 hrs.........			509		25.04	8.42	2.98	·

recovery periods during which the dog was fed and received insulin subcutaneously. The dog was operated on November 10, 1931, and died suddenly without discoverable cause February 9, 1932, 22 days after conclusion of the last experimental period, when it had been long restored to a very lively and apparently satisfactory condition.

During the intervals between experimental periods the dog received twice daily a mixture of 100 gm. of ground dog biscuit, 100 gm. of raw trimmed pancreas, 25 gm. of bone ash, and table scraps equivalent to 20 to 40 calories. Each feeding was followed immediately by subcutaneous injection of 30 units of insulin.

Inspection of Table III discloses that evidence of insulin absorption from the alimentary canal is limited largely to changes in excretion of sugar and nitrogen in the urine. Temporary storage of glycogen was indicated in some instances by elevated D:N ratios of subsequent periods. Considerable decreases in rate of sugar excretion occurred usually without significant variation in blood sugar values. In one period sugar practically disappeared from the urine without appreciable change in the diabetic level of blood sugar (*cf.* the data of January 16, Table III). We offer no explanation of this phenomenon, nor of the persistence during prolonged fasting of high D:N ratios more characteristic of the phlorhizinized dog.

Absorption of insulin occurred when it was given with antitrypsin by stomach tube, and when injected, with or without anti-

trypsin, directly into the lumen[8] of the duodenum. But insulin was not absorbed when given without antitrypsin by stomach tube. The administration of antitrypsin alone had no effect. Apparently the positive effects obtained from stomachal administration of insulin with "antitrypsin" are attributable to antipeptic activity of the antiprotease preparation. We were able to demonstrate *in vitro* the presence of antipepsin in our so called antitrypsin material, although the amount appeared to be relatively small. The observed failure of our antiprotease preparation to improve the absorption of insulin when injected directly into the duodenum of the depancreatized dog is to be expected if the succus entericus contains no protease capable of inactivating insulin, or if the "antitrypsin" extract administered did not contain antienzymes specific for proteases peculiar to the intestinal secretions. There appears to be considerable uncertainty whether any protease not found in pancreatin is secreted by the intestine, and we had anticipated that an antienzyme mixture which would inhibit pancreatic digestion of protein would, *pari passu*, inhibit intestinal proteolysis.

We cannot attribute to destructive action by the liver the relatively small effect of insulin introduced into the duodenum, since Bollman and Mann (12) have shown that insulin injected into the intestinal wall is as effective as when given subcutaneously. We believe that our results discount the practicality of efforts to protect insulin against proteolysis in the alimentary canal, and point more definitely to the physical obstacles to absorption imposed by the protein character of insulin.

[8] Duodenal puncture, even by a fine needle, is open to the criticism that insulin may escape into the wound and thus enter the blood stream. To minimize this danger the following technique was observed. A small needle attached to an empty syringe was introduced through the duodenal wall and duodenal contents aspirated, thus insuring that the point of the needle was within the lumen. While the needle was held in position the empty barrel of a fresh syringe was attached. The insulin mixture was now poured into the syringe and injected, with some air, into the duodenum. In our opinion the possibility of insulin entering the blood stream directly was exceedingly small under these conditions, and does not account for the more pronounced insulin effects observed after duodenal injection as compared with stomachal administration. It may be noted in this connection that insulin effects were entirely nil when the injection was made in identical manner in the dog which retained its pancreas.

SUMMARY

1. A preparation of antitrypsin is described which is at least 90 per cent effective in preventing inactivation of insulin by pancreatin under *in vitro* conditions favorable for tryptic digestion. Apparently a stoichiometric relation holds between trypsin and antitrypsin, since a small amount of antitrypsin will not afford protection against an indefinitely large amount of trypsin.

2. The unique advantages of insulin as an indicator of antienzyme effectiveness are cited.

3. No insulin effects can be observed in the normal dog when mixtures of insulin and antitrypsin are introduced directly into the duodenum, perhaps because of a compensatory decrease in insulin secretion by the pancreas.

4. Insulin administered by stomach tube to depancreatized dogs has no effect upon sugar excretion or D:N ratios. When mixed with antitrypsin and given by stomach tube, insulin causes a marked decrease in sugar output, thus indicating an antipeptic activity of the antitrypsin preparation.

5. Insulin alone or with antitrypsin, introduced directly into the duodenum of the depancreatized dog, will abolish the sugar excretion for short periods and maintain the urine sugar values at low levels for longer periods. Compensatory, high D:N ratios in subsequent periods suggest an interim storage of glycogen.

6. Subcutaneous injection of antitrypsin alone in rabbits and subcutaneous or enteral administration in dogs have no detectable effect upon blood or urine sugar.

7. The relatively small absorption of insulin direct from the intestine, and the failure of our antiprotease mixture to improve its absorption, are probably attributable either to inactivation of the major portion by intestinal enzymes or bacteria, or to inherent physical obstacles to diffusion imposed by the protein character of insulin.

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DIFFUSIBLE SERUM CALCIUM BY HIGH PRESSURE ULTRAFILTRATION*

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Within recent years many investigations have been undertaken to determine the amount of diffusible calcium in blood serum. The principle underlying the methods for the determination of this diffusible fraction is based on the generally accepted fact that part of the serum calcium is in a free state (either ionic or as an unionized salt), whereas the remainder is bound by the serum proteins either by chemical combination or by adsorption. Therefore, a number of methods have been proposed employing the principle that the free calcium ion or salt will diffuse through a collodion membrane, whereas the protein-bound calcium will be withheld due to the impermeability of the membrane to the large protein molecule. A number of investigators have used the process of dialysis for the estimation of diffusible serum calcium, while others have used the principle of ultrafiltration. Also, some investigators believe that the calcium in certain protein-free body fluids (such as cerebrospinal, peritoneal, and edematous fluids) represents diffused serum calcium, and have used the calcium contents of those fluids to represent diffusible serum calcium.

Of these methods, those based on the principle of ultrafiltration seem to yield the best results, although there is some inconsistency in the manner of making the collodion membranes, and in the values obtained for the amount of diffusible calcium in normal blood serum. Cushny (1), Neuhausen and Pincus (2), Tschimber (3), Stewart and Percival (4), and Kirk and King (5) have all used collodion sacs or membranes made from solutions of collodion in either glacial acetic acid or in an ether-alcohol solvent. They filtered the serum at pressures of 120 to 180 mm. of mercury, and the time of filtration varied from 2 to 16 hours. Their results indicate the presence of diffusible calcium to the extent of 40 to

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80 per cent of the total calcium in human serum.   Greenberg and Gunther (6) have recently proposed a method of ultrafiltration in which they give definite directions for the preparation of the collodion sac and the manner of carrying out the ultrafiltration process.   They have used their method in a number of investigations (7) and have established a normal value for diffusible calcium in human serum of 4.2 to 6.8 mg. per 100 cc. of serum (analysis of forty-two samples of blood from twenty-four normal individuals). Based on an average normal serum calcium content of 10 mg., their values would represent from 42 to 68 per cent of the serum calcium to be diffusible.   They also used a negative pressure of 150 mm. of mercury.   Still more recently, Spiegler (8) has used a Bechhold filter impregnated with collodion under a negative pressure of 740 mm. of mercury, employing an ordinary water suction-pump. He does not give any normal values for diffusible calcium by his method.

Since there is some inconsistency in the results obtained by the various methods of ultrafiltration, probably due primarily to differences in the permeability of the collodion sacs, the possibility of using du Pont cellophane No. 300 as a filtering membrane was investigated.   Ever since its appearance on the market this product has been used by colloid chemists as a membrane for dialyzing or ultrafiltering colloidal solutions.   The uniform thickness and permeability of this membrane would at least remove the uncertainties of casting one's own sacs from collodion solutions.   Since cellophane is considerably more impermeable to blood serum than the very thin collodion sacs, it requires considerably more pressure to force the fluid through the membrane.   The question has been raised (2, 4) as to whether this high pressure on the blood serum might cause the decomposition of any labile combination between calcium and the blood proteins, which would give higher values for the amount of diffusible calcium than those results obtained by low pressure methods.

Apparatus

A diagram of the apparatus[1] used is shown in Fig. 1.   It is simply a diminutive form of the usual large size high pressure

[1] The apparatus was machined out of brass in the machine shop of the Mechanical Engineering Department of The Rice Institute under the direction of Mr. R. R. Crookston.

ultrafilter. The filtering chamber has a diameter of 1.5 inches, and a capacity of about 25 cc. The apparatus consists of four parts. *A* is the base of the filtering chamber into which fits the cellophane membrane with its supporting structures; namely, a plate drilled with numerous small holes, over which is placed a silver gauze of about 15 mesh. Over this is then placed a piece of high grade ashless filter paper and finally the cellophane membrane. This whole assembly is supported between two rubber washers. *B* is
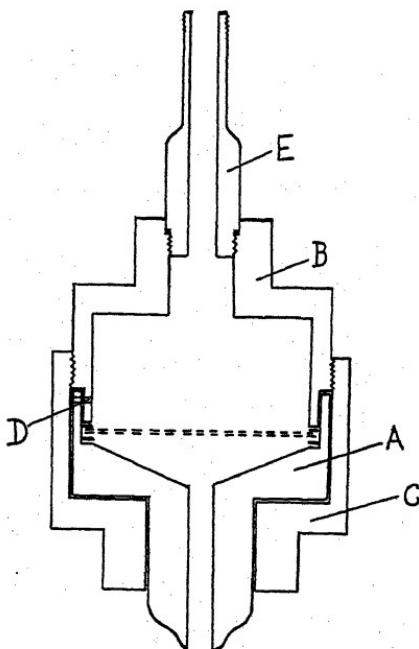
FIG. 1. High pressure ultrafilter for the determination of diffusible serum calcium. See the text for an explanation of the letters.

the upper half of the chamber which fits snugly into the base, and is pulled tightly against the membrane assembly by the union, *C*. Both *C* and *B* are milled hexagonally to receive the tightening wrenches. One feature of the apparatus is the pin and slot arrangement, *D*, which prevents any twisting or tearing of the cellophane membrane while in the process of tightening the union. The pin is set in the upper section of the chamber, and the slot is cut in the wall of the lower section. *E* is composed of an ordinary

tire valve welded to a threaded connection that fits into the upper
end of the chamber.

To use the apparatus, it is first completely assembled (with the
exception of the upper part, E); a measured quantity of the serum
is run into the chamber, and then the valve stem is screwed in and
drawn up tightly against a small rubber gasket. Pressure is
developed in the chamber by attaching the valve stem to a nitrogen
tank through a suitable regulating valve, the actual pressure being
read from the small gage on the regulator. The diagram is
approximately two-thirds actual size.

<div align="center">EXPERIMENTAL</div>

The first experimental work was to determine the uniformity of
the calcium concentration in various portions of the ultrafiltrate,

<div align="center">TABLE I</div>

*Showing Uniformity of Calcium Concentration in Different Portions of Ultra-
filtrate, and Uniformity of Cellophane No. 300 to Calcium Ultrafiltration*

2 cc. fractions were used in every case.

Fluid ultrafiltered	Membrane	Ca in ultrafiltrate			
		Fraction 1	Fraction 2	Fraction 3	Fraction 4
		mg. per 100 cc.	*mg. per 100 cc.*	*mg. per 100 cc.*	*mg. per 100 cc.*
CaCl₂ solution contain-	A	9.5	9.2	9.6	9.3
ing 9.2 mg. Ca per 100	B	9.0	9.2	9.3	9.5
cc.	C	9.1	9.5	9.5	9.2
	D	9.2	9.0	8.9	9.1
Serum containing 11.0	A	6.4	6.3	6.2	6.3
mg. Ca per 100 cc.	B	6.2	6.3	6.5	6.2

and to show the uniformity of cellophane membranes to the ultra-
filtration of calcium. To show these effects, four discs were cut
from various sections of a roll of cellophane, through which 10 cc.
portions of a calcium chloride solution were filtered under 150
pounds pressure. At the same time successive 2 cc. portions of
the filtrate were removed as soon as formed, and analyzed for
calcium (9). The same procedure was used with 15 cc. portions of
serum. The results are given in Table I.

To study the effect of pressure, 10 cc. samples of a water solution

of calcium chloride (containing 9 mg. of calcium per 100 cc.) were
filtered through the membranes under different pressures. After
about 5 cc. of the ultrafiltrate had formed, a 2 cc. aliquot was
removed and analyzed. At the same time a 2 cc. aliquot of the
unfiltered fluid in the chamber was removed and analyzed. In
determining the effect of pressure on the serum the following pro-
cedure was used. 5 cc. portions of serum were put through the
apparatus at different pressures, and the first 2 cc. fraction of the
ultrafiltrate that formed was analyzed. Also, two samples of the

TABLE II

Effect of Pressure on Calcium Determination by Ultrafiltration

Pressure of N_2	CaCl₂ solution containing 9.0 mg. Ca per 100 cc.		Serum containing 10.8 mg. Ca per 100 cc. Ca in ultrafiltrate	Time to filter 2 cc. serum
	Ca in filtrate	Ca above membrane		
lbs. per sq. in.	*mg. per 100 cc.*	*mg. per 100 cc.*	*mg. per 100 cc.*	*hrs.*
50	8.8	9.1	7.7	4½
	9.0	9.0	8.0	
100	9.0	9.1	7.7	3
	8.8	9.0	8.1	
150	9.1	9.0	7.8	2
	8.7	9.0	8.0	
200	9.0	8.9	7.7	1¾
	8.9	9.1	8.0	
150 O_2			7.6	2
			7.9	

same serum were ultrafiltered, oxygen gas at 150 pounds pressure
being used. In this case, the results were identical with those
obtained with nitrogen. The results of these experiments are
given in Table II, the calcium content being expressed in mg. per
100 cc. The approximate time to filter 2 cc. of serum is also given.

Table III gives the normal values for the distribution of diffusible
and non-diffusible calcium as determined by this method. Sera 1
to 11 were from persons who had passed through an ear, nose, and
throat clinic where the usual routine clinical examination had been
made; namely, red and white blood cell count, white blood cell
differential count, hemoglobin, Wassermann, and urine albumin,
sugar, and acetone body determinations. All findings were

normal except as noted.   Sera 12 and 13 were pooled samples obtained from normal students.   It will be seen that the serum calcium varied between 9.6 and 12.9 mg. per 100 cc., with the diffusible calcium ranging from 6.0 to 8.3 mg. per 100 cc.   The percentage of diffusible calcium is quite constant (60 to 67 per cent) with a general average of 64 per cent.

This method therefore gives a higher value for the concentration of diffusible serum calcium, but a more constant ratio between the diffusible and non-diffusible fractions.   This would seem to bear

TABLE III

Distribution of Diffusible and Non-Diffusible Calcium in Serum as Determined by Ultrafiltration

Serum No.	Serum Ca	Diffusible Ca		Remarks
	mg. per 100 cc.	*mg. per 100 cc.*	*per cent*	
1	10.2	6.8	67	Normal
2	11.0	7.2	65	"
3	12.9	8.1	63	Child, albuminuria (trace)
4	12.8	8.3	65	Normal
5	12.1	7.3	60	"
6	12.1	7.7	64	"
7	11.0	7.4	67	White blood cell count 12,000, albuminuria (trace)
8	10.3	6.5	63	Normal
9	11.5	7.3	63	"
10	11.5	7.5	65	"
11	11.0	7.2	65	"
12	9.6	6.0	62	Pooled normal sera
13	10.8	6.7	62	" " "

out the statement of some (2, 4) that a high pressure does tend to decompose any labile combination between the calcium and the protein.   Yet in Table II it was shown that pressures of from 50 to 200 pounds per sq. inch had no effect on this distribution.   Therefore, the value of 36 per cent for non-diffusible calcium as obtained in these normal bloods at 150 pounds pressure, must represent the amount of calcium that is *firmly* bound by the proteins, hence the constancy of this value.   Also, this constancy again supports the statement that cellophane is uniformly permeable to diffusible calcium, as already demonstrated in Table I, which cannot be said

of collodion sacs made under the best controlled conditions. Certainly, the ease of handling the small cellophane discs used in this apparatus as compared to making and preserving collodion sacs, is far greater.

These analyses were all made in the same way. Approximately 5 cc. of serum were placed in the apparatus, and subjected to a pressure of 150 pounds. At the end of 2 hours, a little more than 2 cc. of ultrafiltrate had collected. This time factor is very constant and never varies more than 5 or 10 minutes. A 2 cc. aliquot of the filtrate is analyzed for calcium (9), and the results are expressed as mg. of calcium per 100 cc. of serum, no correction being applied for the volume of proteins present in the original serum.

We are indebted to Dr. Sidney Israel, and to Dr. Violet Keiller of the Hermann Hospital, for the samples of normal blood used in this work.

SUMMARY

1. A small form of a high pressure ultrafilter with du Pont cellophane No. 300 as a membrane, and its application to the determination of diffusible serum calcium is described.

2. With this apparatus the concentration of normal diffusible calcium is somewhat higher than those values obtained by low pressure methods, but more constant. The normal values obtained by this method show 64 per cent of the serum calcium to be diffusible, with the remaining 34 per cent *firmly* and *strongly* held by the proteins.

3. These cellophane membranes are readily available, and are of uniform permeability to calcium. The use of these membranes, therefore, eliminates any uncertainty of permeability that is inherent in cast collodion sacs, due to the use of various preparations of collodion and methods of making them.

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THE DETERMINATION OF BLOOD GLUTATHIONE

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In discussions of the factors necessary for accuracy in blood glutathione determinations, far more consideration has been given to the method of estimation than to the type of blood filtrate used. It has been shown that either iodometric titration, under the proper conditions of pH, temperature, and potassium iodide concentration, or ferricyanide colorimetric estimation accurately determines glutathione in pure solution. Mason (1) has further shown that both methods agree fairly well when applied to the same blood filtrate. However, the use of different filtrates combined with various methods of estimation has led to varying results and, therefore, much controversy over the actual amount of glutathione present in blood.

Two essential requirements for the blood filtrate are (1) that no reduced glutathione be lost by autoxidation, and (2) that the glutathione be completely recovered from the blood. In the colorimetric ferricyanide method developed by Mason (1) a tungstic acid blood filtrate is used. This method has recently been applied to human blood by two investigators, Downes, reported by Benedict (2), and Schelling (3). The results of both investigators would indicate that the blood glutathione is considerably less than generally supposed. According to the original intention of Folin and Wu (4) for the tungstic acid filtrate, 1:10 dilution of the blood, it is only slightly acid. The pH is about 5.5, varying slightly with each individual blood. With Schelling's modification, 1:5 dilution, the pH is about 4.9. It is a well known fact that glutathione undergoes autoxidation at a neutral or alkaline pH and even at slight acidity. Mason realized the possibility of autoxidation in such filtrates and in a later paper (5) sug-

gested a modification of the Folin-Wu filtrate, which increased the acidity. Experiments which we have carried out on tungstic acid filtrates of varying pH show that considerable reduced glutathione is rapidly lost from the slightly acid tungstic acid filtrates by autoxidation. The values reported by Downes and Schelling are, therefore, undoubtedly low because of autoxidation which has occurred during preparation of the tungstic acid filtrate.

The trichloroacetic acid filtrate which was first used for gluta-thione determinations by Tunnicliffe (6) would be expected to prevent autoxidation due to its acidity. There is, however, a slight autoxidation in this acid, as shown by Bierich and Kalle (7), causing a loss of 5 per cent in 3 hours in a 10 per cent trichloroacetic acid filtrate. This slow autoxidation could not cause appreciable error if the determination were made without delay. However, the findings of Gabbe (8), Gulland and Peters (9), and of Schelling (3) that there is less sulfhydryl in trichloroacetic than in tungstic acid filtrates of blood indicate that the glutathione is incompletely extracted by this acid and that such a filtrate is not suitable for blood glutathione determinations. While we have not carried out extensive investigations of the trichloroacetic acid filtrate, quantitative determinations on this filtrate compared to a new filtrate to be described showed considerably less sulfhydryl.

In the course of the present work, sulfosalicylic acid has been found to give a protein-free filtrate which fulfils both of the above requirements. No autoxidation has been observed in a 2 per cent sulfosalicylic acid filtrate for several hours, and after 24 hours only a slight loss of sulfhydryl. It also allows 100 per cent recovery of glutathione added to blood before precipitation of the proteins. Other advantages of this filtrate are that filtration is rapid, there is a large yield of filtrate, and, due to its acidity, it is applicable directly to a determination of the oxidized fraction of blood glutathione by the usual nascent hydrogen reduction method.

Our method for the determination of blood glutathione involves the use of a modified iodate procedure in connection with the 2 per cent sulfosalicylic acid filtrate. The method consists of titration of 10 cc. of the filtrate with 0.001 N potassium iodate at 20° in the presence of excess potassium iodide, with starch as an internal indicator. This procedure has been found to be more convenient than the ferricyanide colorimetric method, due to the much

smaller quantities of blood and time necessary for a determination, factors which must be taken into consideration when numerous determinations are to be made. The reduced glutathione value may be obtained within ½ hour after the drawing of the blood, although immediate titration is not necessary. 3 cc. of blood give enough filtrate for a determination of both the reduced and oxidized glutathione.

The original iodometric procedure as developed by Tunnicliffe (6) was a direct titration method with iodine on a trichloroacetic acid filtrate. Most workers have preferred a modification of this method which employed back titration with thiosulfate after addition of excess iodine. However, the results of Delaville and Kowarski (10) and Chang and Ling (11) show the dangers of back titration. These authors have indicated the presence in filtrates of substances with varying speeds of iodine reaction, and have shown higher values to be obtained when the iodine was in contact with the solution for greater lengths of time. We, therefore, desired to avoid the use of a back titration method.

The method developed is a modification of the Okuda (12) iodate titration for cysteine which Hess (13) applied to glutathione. The use of iodate, by which iodine is liberated in solution, is more advantageous than iodine as the standard for titrating, since iodate solutions are stable and repeated standardization is not necessary. Standardization of iodate against glutathione, as suggested by Hess, is also unnecessary. For the estimation of the reduced glutathione Hess used 10 cc. of a molybdic acid blood filtrate, to which were added hydrochloric acid and potassium iodide, titrating at 20° with $M/1200$ (0.005 N) potassium iodate to the yellow iodine end-point. In attempting to use this method, we were not able to obtain an end-point of which we were at all certain; nor could we obtain blood glutathione values which approached the range reported by Hess. It was apparent that considerable error could be introduced by overestimating the end-point through use of iodate as strong as 0.005 N, accompanied by the indefiniteness of the yellow iodine end-point. Accordingly we have used a more dilute iodate, 0.001 N, for titrating, with starch as an internal indicator. During the titration we have maintained the original specifications for temperature. The necessity for temperatures 25° or below has been recently emphasized by King and Lucas (14).

An investigation of substances which might influence the iodine uptake of glutathione has led to the conclusion that under the conditions of the method described, no known blood constituent can interfere except thioneine. This substance, like glutathione, contains a sulfhydryl group and would be expected to act similarly toward iodine. However, thioneine has been found to differ considerably from glutathione. At an extremely acid pH, such as is used in our method, thioneine alone is oxidized but slightly by iodine, while with increasing pH its oxidizability is augmented. In the presence of glutathione, even at very acid pH, the oxidation of thioneine is induced, depending upon the ratio of the amounts of the two substances present. In proportions such as found in human blood, about 60 per cent of the thioneine is reacting. Since normal human blood contains an average of about 5 mg. per 100 cc. (Behre and Benedict (15), 7.5 mg.; Hunter (16), calculated to whole blood, about 5 mg.; Salt (17), calculated to whole blood, 3.8 mg.), the average thioneine error in blood glutathione figures by our method will be about 3 mg. per cent.

In this respect, Mason (1) believed his ferricyanide method more specific than iodine. Slight discrepancies between the two methods in the case of blood he attributed to thioneine. However, our results show that the oxidation of thioneine by ferricyanide may also be induced by glutathione, and in blood filtrates the error produced by thioneine is probably of about the same order with either method.

By the new method the reduced glutathione of venous blood has been found to vary, in a series of thirty normal individuals, from 25 to 41 mg. per 100 cc. of blood, with the average at 34 mg.; in five cancer cases, from 26 to 36 mg. per 100 cc. After zinc reduction this value is increased, indicating the presence of 3 to 11 mg. per cent of oxidized glutathione. That this increase is due to oxidized glutathione has been recently doubted by Schelling (3). However, in the absence of further information on this subject, we are including our figures as oxidized glutathione.

<div align="center">EXPERIMENTAL</div>

Autoxidation in Tungstic Acid Filtrates—The amount of reduced glutathione present in a series of tungstic acid blood filtrates has been estimated by the Mason (1) method at 1, 2, and 3

hour intervals after precipitation of the blood proteins. Due to the slow filtration, it was not possible to obtain enough filtrate for a determination earlier than 40 minutes to 1 hour after the precipitation. The determinations by Mason's method were carried out on 10 cc. of a 1:5 dilution filtrate, or 15 cc. of a 1:10 dilution, made to volume in a 25 cc. volumetric flask; thus, in the case of the former, the concentration was the same as Schelling's (3), but half as much filtrate was used. For comparison the value has also been determined in sulfosalicylic acid filtrates by the iodo-

TABLE I

Effect of Time and pH on Glutathione Value of Tungstic Acid Filtrates

Blood No.	Source of blood	GSH* found in sulfosali- cylic acid filtrate	GSH found in tungstic acid filtrate after			pH of tungstic acid filtrate	Type of tungstic acid precipitation
			1 hr.	2 hrs.	3 hrs.		
		mg. per cent	*mg. per cent*	*mg. per cent*	*mg. per cent*		
1	E. S.	35.1			6.6	5.6	Folin-Wu (4)
2	S. H.	29.5		13.8		5.5	"
3	C. A.	30.7		17.0		5.5	"
4	J. D.	32.4	22.9	14.6	12.4	5.1	Schelling (3)
5	A. A.	38.0	40.3	25.3	20.4	4.9	"
6	E. S.	35.0		20.6	15.0	4.5	"
7	"	35.0	26.9		23.1†	†	"
8	A. A.	38.0		28.7	21.8	3.0	Mason (5)
9	J. K.	33.4	29.4			2.2	New
10	Mixed	30.7	26.5			2.0	"

* GSH denotes reduced glutathione.

† The pH during filtration was 4.5. The filtrate was acidified to pH 2.0 at the time of the first determination by adding 1 drop of concentrated sulfuric acid. The filtrate stood at this pH until the second determination.

metric titration to be described in a later section. An attempt to apply the colorimetric estimation to this filtrate failed, as a slight color was produced on neutralization.

It will be observed in Table I that at each succeeding hour the glutathione found in tungstic acid filtrates was less, and the longer the filtrate stood the greater was the difference between the values found in the tungstic and sulfosalicylic acid filtrates, in which the reduced glutathione value was found constant for several hours. The loss in tungstic acid filtrates which was undoubtedly due to

autoxidation was even observed when the acidity of the filtrate was increased to pH 3.0 and 2.0 (Bloods 8 and 7, respectively). Since autoxidation was considerably retarded at pH 2.0, an attempt was made to prepare a tungstic acid blood filtrate which had this pH from the time of precipitation of the proteins. To 1 volume of blood were added 1.5 volumes of water, followed by 1.5 volumes of $\frac{2}{3}$ N sulfuric acid and 1 volume of sodium tungstate. The filtrate itself was clear, but in two attempts, after development of the blue color, the solution was so cloudy that it was very difficult to obtain accurate colorimeter readings. In our experience this effect seems to be related to the increased amount of salt

TABLE II

Recovery of Glutathione Added to Blood before Tungstic Acid Precipitation of Proteins

Blood No.	GSH added	GSH found after		Apparent GSH recovered after		pH of filtrate
		1 hr.	2 hrs.	1 hr.	2 hrs.	
	mg. per cent	*mg. per cent*	*mg. per cent*	*mg. per cent*	*mg. per cent*	
1	0	26.2	22.0			4.7
	20	44.0	40.0	17.8	18.0	4.7
2	0	19.8	13.2			4.6
	30	52.8	31.5	33.0	18.3	4.5
		2 hrs.	3 hrs.	2 hrs.	3 hrs.	
3	0	10.9	7.4			4.9
	30	46.1	36.8	35.2	29.4	4.8

present after neutralizing the more acid filtrates. Approximate readings were taken, however, and these values (Bloods 9 and 10) more closely approached the values for the sulfosalicylic acid filtrates.

The autoxidation was also apparent in recovery experiments when the glutathione was added to blood before precipitation of the proteins. In this case, the added glutathione was in the slightly acid tungstic solution during the time of filtration. Determinations on the original filtrates and on those containing the added glutathione were made as soon as possible and again after 1 or 2 hours. Each time, as seen in Table II, there was a loss in both filtrates, although the apparent glutathione recovered did not

always change to any great extent. This figure has no significance as to actual recovery, since the filtrates were made separately and the rate of autoxidation in individual filtrates was exceedingly irregular, as may be observed in both Tables I and II.

When, however, the glutathione was added to the filtrate and the determinations made immediately, the added glutathione was 100 per cent estimated. Such an experiment is recorded in Table III.

Sulfosalicylic Acid As Protein Precipitant[1]—It was first necessary to investigate the conditions under which sulfosalicylic acid could best be used as a precipitant for blood proteins. It was found necessary to lake the blood with water before addition of acid. 10 per cent sulfosalicylic acid filtrates were cloudy and slightly pink in color. However, providing the blood has been laked properly,

TABLE III

Recovery of Glutathione Added to Tungstic Acid Filtrate of Blood

GSH added	GSH found	GSH recovered
mg. per cent	*mg. per cent*	*mg. per cent*
0	35.0	
20	55.5	20.5
30	64.9	29.9
40	75.2	40.2

a filtrate approximating 2 per cent acid (with pH below 2.0) is clear, free from protein, and of the proper acidity for use in the iodate titration for reduced glutathione.

The laking process may be thoroughly accomplished in a few minutes, but the question arises, is there any loss of sulfhydryl during this time through autoxidation on standing in the neutral or slightly alkaline solution? Filtrates of blood laked with water from 2 minutes to 1 hour were compared as to their sulfhydryl content with a similar sample laked 10 minutes with a hypotonic (0.01 N) oxalic acid solution. When the blood used was normal venous blood, the same amount of sulfhydryl was found in all samples regardless of whether laked with acid or with water from 2 minutes up to 1 hour. This is admittedly surprising, as the pH

[1] We are indebted to Dr. W. C. Hess for suggesting the use of this acid to

of the laked blood would be expected to be about neutral, a pH at which autoxidation of glutathione readily occurs in pure solution. However, when a mixture of arterial and venous sheep blood was used, there was a steady decrease in sulfhydryl content the longer it was laked with water. Since arterial blood contains a larger percentage of oxidized glutathione, the behavior of this blood as distinguished from the venous blood under the same conditions may possibly be explained by the experiments of Harrison (18) in which he points out the property of the disulfide form to act as a catalyst for the oxidation of sulfhydryl at pH 7.4. The rate of oxidation depended upon the amount of disulfide present.

Autoxidation in the resulting sulfosalicylic acid filtrate was also investigated. No change in the iodate titration was observed when repeated after 4 or 5 hours; in some filtrates there was no change during 24 hours. Where there was a decrease it was not over 6 per cent in 24 hours.

Procedure for Reduced Glutathione in Blood—As soon as possible after venipuncture, lake thoroughly 1 volume of a well mixed, oxalated[2] sample of blood with 8 volumes of distilled water to allow all of the glutathione to escape from the disintegrated corpuscles. After 5 to 10 minutes, add very slowly with shaking 1 volume of a molar (22 per cent)[3] solution of sulfosalicylic acid. Shake and filter through a dry filter paper. From 3 cc. of blood, 22 to 25 cc. of filtrate will usually be obtained. This filtrate will contain about 2 per cent sulfosalicylic acid due to loss of a slight amount during protein precipitation, probably by adsorption upon the proteins, and will have a pH of 2.0 or below.

Titrate 10 cc. aliquots of the filtrate in a 50 cc. Erlenmeyer flask as follows: After addition of 2.5 cc. of 4 per cent sulfosalicylic acid, 2.5 cc. of 5 per cent potassium iodide containing no trace of free iodine, and 2 drops of 1 per cent starch,[4] run in with care from

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[2] 0.01 cc. of a 30 per cent solution of $K_2C_2O_4$ (3 mg.) is sufficient to oxalate 1 cc. of blood. Cloudy filtrates result from overoxalated blood.

[3] *Sulfosalicylic Acid*—On account of its deliquescence it was necessary to weigh 25 gm. instead of 22 gm. to prepare 100 cc. of a molar solution. The 4 per cent solution may be made by diluting 45.6 cc. of this to 250 cc.

[4] *Starch*—A 1 per cent solution of soluble starch in water or saturated sodium chloride may be used.

a micro burette 0.001 N potassium iodate[5] made up in 2 per cent sulfosalicylic acid (to maintain the desired acidity) until the first blue color persists. During the titration the flask is placed in a beaker of water adjusted to a temperature of 19–20°, which not only maintains the desired temperature for the reaction, but also facilitates the detection of the blue end-point. A white background should be used. This end-point is very sharp, and, when one becomes accustomed to it, will not be overestimated by more than 1 drop. When a blank is run with 10 cc. of 2 per cent sulfosalicylic acid, this is found negligible, only a drop or fraction of a drop, according to the individual, being necessary to produce enough blue color to be easily distinguishable.

The calculation is as follows:

$$\frac{\text{Cc. } 0.001\,\text{N KIO}_3 \text{ (for 10 cc. filtrate)}}{3.26 \text{ (theoretical titer for 1 mg.)}} \times 100 = \text{mg. GSH per 100 cc. blood}$$

TABLE IV

Recovery of Added Glutathione

Blood No.	Blood	GSH added	GSH content of blood	Total GSH found	GSH recovered
		mg. per cent	*mg. per cent*	*mg. per cent*	*mg. per cent*
1	Normal whole blood	100	39.7	138.5	98.8
				136.8	97.1
2	Cancer " "	100	29.8	129.5	99.7
				130.1	100.3

When 1 mg. of pure glutathione in 10 cc. of 2 per cent sulfosalicylic acid is titrated according to the above procedure, the theoretical titer is obtained.

Recovery of Reduced Glutathione Added to Blood—Glutathione added to blood before precipitation of the proteins was 97 to 100 per cent recovered by the new procedure. Table IV records the results.

[5] *Potassium Iodate*—0.1783 gm. weighed exactly and made up to 1 liter with water in a volumetric flask gives a 0.005 N solution. This keeps indefinitely. The 0.001 N solution used in the titration is made by combining 50 cc. of this and 22.8 cc. of the molar sulfosalicylic acid and diluting to 250 cc. in a volumetric flask. Since this loses its strength slowly, a fresh solution should be made from the stock solution once a week or the factor determined by comparing the thiosulfate titration of 10 cc. of the 0.001 N iodate with that of 2 cc. of the 0.005 N iodate.

Control Experiments on Reduction of Oxidized Glutathione—
Solutions of pure reduced glutathione were made of known concentration. A sample was immediately checked for its sulfhydryl content by the iodate titration method. Another sample was buffered to pH 7.0 and partially oxidized by bubbling oxygen through for short periods of time. The amount of sulfhydryl left was determined by iodate titration. Aliquots of this solution, made to 2 per cent acidity with sulfosalicylic acid and treated with zinc as described in the next section, showed complete reduction back to the sulfhydryl form in 20 minutes. Such experiments are recorded in Table V.

TABLE V

Reduction of Oxidized Glutathione by Zinc with Time

Sample No.	Original total glutathione content	Reduced glutathione found					
		After oxygenating	After reduction by zinc for				
			5 min.	10 min.	15 min.	20 min.	30 min.
	mg. per cent	*mg. per cent*	*mg. per cent*	*mg. per cent*	*mg. per cent*	*mg. per cent*	*mg. per cent*
1	100	56	95	96	96	101	
2	100	3	96	95	95	101	101
3	·101	65				100	
4	101	0				101	

*Procedure for Total and Oxidized Glutathione in Blood—*If 3 cc. of blood are deproteinized as described in a preceding section, usually enough filtrate is obtained for a determination of both the reduced and total glutathione. To the 12 to 15 cc. remaining after removal of 10 cc. for the reduced glutathione determination, add 30 to 40 mg. of zinc dust. After a trial weighing the amount may be approximated in further analyses. After allowing to react at room temperature for 20 minutes, remove the excess zinc by filtering through a dry filter paper. Thus, the original concentration of the blood filtrate (1:10 dilution of the blood) is maintained. The pH of the filtrate after this treatment is not over 2.2. Measure out 10 cc. and titrate for sulfhydryl as in the original determination of the preformed reduced glutathione. This titration expresses the total glutathione present. The oxidized fraction is obtained by difference between the two titrations.

Interfering Substances—Of the known constituents of the blood, certain of these have been shown by previous investigators not to interfere in iodometric methods. *Urea, glucose, fructose,* and *creatinine* were shown by Tunnicliffe (6) not to react. Kuhnau (19) stated that *uric acid* did not bind iodine in strongly acid solution and showed that *acetoacetic acid* did not except when in contact with iodine for long periods of time. Fourteen *amino acids* tried by Okuda (12) had no effect.

Phenols—Since information on the interference of phenols, with the exception of adrenalin (19), was lacking, we tried the effect of several of these in the present method. All of those tried, phenol, *p*-aminophenol, *p*-nitrophenol, adrenalin, and hydroquinone, gave negative results, both alone and in the presence of glutathione.

Thioneine[6]—Due to the common SH— group, the behavior of this substance has been assumed by most authors to be identical with that of glutathione. However, our experiments show a distinct difference in the reducing intensity of these two sulfhydryl compounds. The amount of iodine consumed by pure thioneine in 2 per cent sulfosalicylic acid solution was almost negligible. Investigation of this reaction at different acidities (Table VI) revealed greater iodine consumption with increasing pH. At pH 5.9 almost the theoretical amount was taken up.

When thioneine was added to 2 per cent sulfosalicylic acid blood filtrates, an acidity at which it consumed practically no iodine in pure solution, an entirely different situation was found. The thioneine took up considerably more iodine, which was not a constant amount when equal amounts of thioneine were added to different blood filtrates. These variations correlated roughly to the variations in glutathione content of the blood (Table VII). Since this suggested that glutathione was causing an induced oxidation of thioneine by iodine, its effect on thioneine in pure solution was tried. It was found that in 2 per cent sulfosalicylic acid an induced oxidation did occur, the magnitude of which depended upon the relative amounts of the two substances. This effect is, however, much smaller in the blood filtrate than in pure solution.

Since it had previously been found that the acidity of the medium

[6] We are indebted to Dr. S. R. Benedict and to Dr. T. B. Johnson for samples of thioneine used in this study.

TABLE VI

Effect of Increasing Acidity on Thioneine Titration

pH	Buffer or acid	Titration of 1 mg TSH* with 0.001 N KIO₃
		cc.
5.9	Na₂HPO₄-KH₂PO₄	4.15†
4.8	Na₂HPO₄-HCl	2.80
4.5	"	2.45
4.3	"	2.14
3.5	"	0.63
Below 1.2	0.7 per cent sulfosalicylic acid	0.50
" 1.2	1.67 " " " "	0.30
" 1.2	2.0 " " " "	0.26
" 1.2	10.0 " " trichloroacetic acid	0.44
" 1.2	0.04 " " HCl	0.55
" 1.2	0.08 " " "	0.40
" 1.2	0.2 " " "	0.30
" 1.2	2.0 " " "	0.15
" 1.2	2.8 " " "	0.10
" 1.2	5.0 " " "	0.10

* TSH denotes reduced thioneine.

† For this pH it was necessary to use 0.001 N I₂. Theoretical titer for 1 mg. of thioneine = 4.36 cc.

TABLE VII

Relation between Amount of Glutathione Present and Per Cent of Added Thioneine Oxidized

GSH present	TSH added	Ratio	TSH oxidized	Type of solution
mg. per cent	mg. per cent		per cent	
30	5	600:100	60	2 per cent sulfosalicylic acid blood filtrate
37	50	74:100	35	2 " " " " " "
39	100	39:100	31	2 " " " " " "
22	100	22:100	15	2 " " " " " "
15	100	15:100	13	2 " " " " " "
50	25	200:100	69	2 per cent sulfosalicylic acid
50	50	100:100	63	2 " " " "

controlled the iodine uptake of thioneine alone, the effect of pH on the induced oxidation of thioneine was investigated. Here,

however, the acidity of the medium had but little effect (Table VIII). When a 1:1 proportion of the two substances was maintained, varying the acidity from pH 1.0 to 4.0 did not increase the catalytic oxidation of the thioneine, which was about 63 per cent in this range. At pH 5.9 the thioneine was completely oxidized in the presence of an equal weight of glutathione.

TABLE VIII

Effect of Glutathione on Thioneine Titration at Varying pH (Proportion 1:1)

| pH | Buffer or acid | Titration with 0.001 N KIO₃ | | | Differ-ence due to TSH | TSH oxi-dized |
		0.5 mg. GSH	0.5 mg. TSH	0.5 mg. each		
		cc.	*cc.*	*cc.*	*cc.*	*per cent*
About 1.0	1.67 per cent sulfosalicy-lic acid	1.69	0.15	3.04	1.35	61.9
1.8	Na₂HPO₄-HCl	1.67	0.30	3.05	1.38	63.3
2.7	KH₂PO₄-HCl	1.72	0.39	3.04	1.32	60.6
3.4	"	1.77*	0.85*	2.90*	1.13	51.8
3.9	"	1.70*	1.10*	3.13*	1.43	65.5
5.9	Na₂HPO₄-KH₂PO₄	1.69*	2.27*	3.89*	2.20	101.0

* 0.001 N I₂ used for these titrations as solution was not acid enough to liberate iodine when KIO₃ was used.

TABLE IX

Effect of Glutathione on Oxidation of Thioneine by Ferricyanide

| Amount of | | Ratio | Glutathione value by Mason's method | | | |
GSH	TSH		GSH alone	GSH + TSH*	Increase due to 0.5 mg. TSH	
mg.	*mg.*		*mg. GSH*	*mg. GSH*	*mg. GSH*	*per cent GSH*
0.5	0.5	1:1	0.51	0.75	0.24	48
1.0	0.5	2:1	1.01	1.29	0.28	56

* Thioneine alone gave too little color for a reading. It was almost negative.

The effect of glutathione in catalyzing the oxidation of thioneine by ferricyanide was also investigated, with similar results (Table IX). Mason's method (1) was used. Thioneine alone gave only very slight reaction, but in the presence of glutathione it was oxidized. When the proportion of glutathione to thioneine was 1:1,

thioneine gave an amount of color equivalent to 48 per cent of glutathione; when the proportion increased to 2:1, the equivalent became 56 per cent. This catalytic effect of glutathione was not reported by Mason, who stated that thioneine produced only one-tenth as much color as an equal weight of glutathione. However, the figures in Tables IX and VII show that the oxidation of thioneine in the presence of increased amounts of glutathione is about the same with ferricyanide as in our iodometric procedure.

When glutathione and thioneine are present in a blood filtrate in the proportion of 6:1, which is approximately as found in blood, the thioneine is 60 per cent oxidized by iodine. This means that if 5 mg. of thioneine are present in blood an error of about 3 mg. will be found in the glutathione figures.

Unsaturated Fatty Acids—In consideration of Chang and Ling's (11) suggestion that unsaturated fatty acids are extracted from tissue by trichloroacetic acid, we investigated the effect of sulfosalicylic acid on the extraction of these substances from blood. If any unsaturated fatty acids were present in the resulting blood filtrate, they could be extracted from this by a suitable solvent. Hexane was chosen because it not only is a good solvent for fatty acids, but is also immiscible with water. It was first determined that hexane extraction of a standard solution of pure glutathione in 2 per cent sulfosalicylic acid did not change its titer. Next 2 per cent sulfosalicylic acid, shaken with oleic acid, was extracted. It consumed no iodine either before or after extraction, indicating either that 2 per cent sulfosalicylic acid did not take up any of this unsaturated fatty acid or that it was not reacting with iodine. However, since glutathione can act as catalyst for the oxidation of unsaturated fatty acids (Hopkins (20)), the same experiment was tried in the presence of glutathione. The titer of the glutathione standard was not increased by shaking with oleic acid, and this remained the same after extraction with hexane. It was apparent that none of this unsaturated fatty acid was taken up by the sulfosalicylic acid. Finally, sulfosalicylic acid filtrates of normal blood were extracted with hexane. There was no difference in the titration before and after extraction. From this it was concluded that no unsaturated fatty acids had been extracted from normal blood by 2 per cent sulfosalicylic acid and, therefore, did not contribute toward the glutathione values as reported in this paper.

Results on Blood

Reduced Glutathione—Glutathione values obtained in venous blood of thirty normal individuals from 20 to 40 years of age are listed in Table X.

TABLE X

Reduced Glutathione Values in Normal Venous Blood

Blood No.	Individual	Age	Sex	R.b.c.	Hb (Sahli)	Color index	GSH	Gabbe's quotient $\frac{GSH}{R.b.c.}$	GSH × Hb R.b.c.
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)
		yrs.		*millions*	*per cent*		*mg. per cent*		
1	D. M.	20	F.	5.0	103	0.97	40	8.0	8.2
2	A. S.	23	"	4.5	86	1.05	41	9.1	7.8
3	A. A.	31	M.	5.1	105	0.97	38	7.5	7.8
4	A. M.	24	"	5.4	101	1.07	41	7.6	7.7
5	J. S.	26	F.	4.6	87	1.06	38	8.3	7.2
6	"	26	"				37		
7	A. H.	31	"	4.2	82	1.02	36	8.6	7.0
8	W. W.	24	M.	5.4	105	1.03	36	6.7	7.0
9	L. R.	23	"	5.5	99	1.11	37	6.7	6.7
10	J. K.	23	"	5.2	105	0.99	33	6.3	6.7
11	E. F.	25	F.	4.8	95	1.01	34	7.1	6.7
12	G. W.	30	"	4.6	93	0.99	33	7.2	6.7
13	"	31	"				39		
14	E. S.	23	"	4.6	84	1.10	35	7.6	6.4
15	J. F.	21	M.	5.4	101	1.07	34	6.3	6.4
16	R. E.	26	F.	5.1	94	1.08	34	6.7	6.3
17	J. M.	40	M.	6.1	105	1.16	36	5.9	6.2
18	E. S.	27	"	5.8	103	1.13	35	6.0	6.2
19	"	27	"				41		
20	"	27	"				35		
21	E. H.	24	"	5.3	114	0.93	29	5.5	6.2
22	A. G.	20	F.	4.7	96	0.98	30	6.4	6.1
23	A. L.	23	"	4.8	96	1.00	30	6.2	6.0
24	C. A.	25	M.	5.4	98	1.10	31	5.7	5.6
25	S. H.	28	"	5.4	104	1.04	29	5.4	5.6
26	W. H.	38	"	5.1	110	0.93	25	4.9	5.4
27	E. M.	23	F.	4.4	90	0.98	26	5.9	5.3
28	G. B.	26	M.				39		
29	J. D.	26	"				32		
30	M. C.	24	F.		100		33		
Average..................................							34		

The values cover the range 25 to 41 mg. of reduced glutathione per 100 cc. of blood, with the average at 34 mg. There is no particular correlation between the amount of glutathione and either the age or sex, although the range of age is rather limited. Since the glutathione is contained only in the corpuscles, Gabbe (8) calculated the quotient, $\dfrac{\text{GSH}}{\text{R.b.c.}}$, giving a figure which expresses the glutathione content of the red blood cell per unit number of cells.

TABLE XI

Total and Oxidized Glutathione in Blood

Blood No.	Individual	Diagnosis	Glutathione found		
			Total	Reduced	Oxidized
			mg. per cent	*mg. per cent*	*mg. per cent*
1	G. W.	Normal	46	39	7
2	J. M.	"	45	36	9
3	A. S.	"	46	41	5
4	J. D.	"	43	32	11
5	E. S.	"	43	35	8
6	"	"	40	35	5
7	J. K.	"	37	33	4
8	S. H.	"	37	29	8
9	C. A.	"	40	31	9
10	A. A.	"	47	38	9
Average.............................			42		
11	M. O.	Carcinoma, breast	36	30	6
12	M. M.	" "	32	29	3
13	L. R.	" rectum	36	26	10
14	E. R.	" antrum	42	31	11
15	P. F.	Epithelioma, lip	40	36	4
Average.............................			37		

Such a calculation has been applied to our figures (Column 9, Table X), giving values which cover the range 4.9 to 9.1. In Bloods 21 and 26, where the hemoglobin is too high per unit of red blood cell (Color index), the glutathione is low. It seems logical, therefore, to incorporate the hemoglobin into Gabbe's formula. When this is done by calculating the quotient, $\dfrac{\text{GSH} \times \text{Hb}}{\text{R.b.c.}}$, a more

constant value is obtained (Column 10, Table X). The figure obtained should express roughly the oxidizing power of the red blood cell per unit number of cells. The constancy of this value suggests that there may be a compensatory relationship between the amount of hemoglobin and the glutathione in the blood, or that the hemoglobin may determine that fraction of glutathione which occurs in the reduced form in venous blood.

Total and Oxidized Glutathione—In Table XI are listed comparative values for total, reduced, and oxidized glutathione on ten normal individuals and five cancer cases.

Increases over the reduced glutathione figure have been obtained by zinc reduction and subsequent titration, with an apparent oxidized glutathione figure of 4 to 11 mg. per cent in the case of normals and 3 to 11 mg. per cent in the cancer cases. The total glutathione found in normal venous blood covers the range 37 to 47 mg. per cent. Due to the small number of cancer cases, definite conclusions cannot be drawn, although it seems that both the total and reduced glutathione figures fall in the lower part of the normal range.

<div align="center">SUMMARY</div>

1. Slightly acid tungstic acid blood filtrates have been shown unsuitable for blood glutathione determinations due to the rapid loss of sulfhydryl by autoxidation.

2. A method for determination of blood glutathione is described. It depends upon preparation of a sulfosalicylic acid blood filtrate and estimation of the glutathione in this filtrate by titration with 0.001 N potassium iodate.

3. Reduced glutathione values, in a series of thirty normal individuals, were found to cover the range 25 to 41 mg. per 100 cc. of blood, with the average at 34 mg.; in five cancer cases, values of 26 to 36 mg. were found.

4. Zinc reduction of the filtrate gives an increase in this value of 3 to 11 mg., which is assumed to be oxidized glutathione.

5. The only known blood constituent found to interfere in the method was thioneine. This is estimated to produce an error of about 3 mg. in the glutathione values.

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ISOLATION AND IDENTIFICATION OF ERGOSTEROL AND MANNITOL FROM ASPERGILLUS FISCHERI*

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INTRODUCTION

Sterols are widely distributed in nature. It has been suggested that they be classified into three groups according to their occurrence: (1) zoosterols, the animal sterols (cholesterol, coprosterol); (2) phytosterols, the plant or vegetable sterols (sitosterol, stigmasterol); and (3) mycosterols, the sterols found in fungi (ergosterol, fungisterol). Although ergosterol has been detected in small amounts in both animals and higher plants, it is the principal sterol present in fungi.

The physical constants reported by various investigators (1–6) for ergosterol isolated from molds are not in agreement. For example, melting points range from 135–161° and specific rotations vary from −117° to −143.3°. These discrepancies are probably due mainly to the presence of small amounts of impurities (including possibly other sterols) from which it is difficult to separate the ergosterol and some of which may have been actually produced during the attempted purification.

Lack of sufficient material has also handicapped the purification. In most cases the quantity of sterol isolated and actually in hand for identification purposes has been small. As a consequence many reports contain insufficient data for a satisfactory characterization of the sterol. Of the six papers recording the presence of ergosterol in molds, only three (2, 4, 6) give the melting points and optical rotations of both the free sterol and a derivative thereof. In spite of this lack of data, the constants taken as a

whole indicate the presence of ergosterol in representative species of three large groups of molds, *Aspergilli*, *Penicillia*, and *Mucorineæ*.

In a previous investigation (7), the presence of ergosterol in ten different molds, grown on a synthetic medium, was demonstrated by the development of antirachitic potency in the molds on irradiation, but no ergosterol was actually isolated. The present paper deals with the isolation and identification of ergosterol and mannitol from *Aspergillus fischeri* (Thom 5041) grown on an inorganic salt medium containing 15 per cent glucose as the source of carbon.

<div align="center">EXPERIMENTAL</div>

Culturing of Mold—*Aspergillus fischeri* (Thom 5041) was grown in aluminum pans in two large sterilizer-incubators which were kept in a constant temperature room. This apparatus and the procedure used for culturing molds on a large scale in the laboratory will be described in more detail in a forthcoming paper.

The medium on which the mold was grown contained the same inorganic salts as were used in our previous work (7). However, to obtain a greater yield of mycelium the concentration of glucose was increased to 15 per cent, and in order to insure complete sterilization at the temperature available (96–100°) the medium was acidified with phosphoric acid to pH 2.5. Just previous to inoculation, the medium was adjusted to pH 6.8 by means of sterilized potassium hydroxide solution.

Incubation for 10 days at 30–35° resulted in the fermentation of about 80 per cent of the glucose and the formation of a thick mat of mycelium. The fermented liquor was siphoned off, and the mats were washed with water to remove adhering medium, steamed at 99° for 1 hour to kill the spores, dried at 65° for 3 days, and then finely ground.

Isolation of Ergosterol—18 kilos of the dried, ground mold, in batches of 1 kilo each, were extracted four times with 96 per cent ethyl alcohol on a warm sand bath. The alcohol extracts were decanted and the moist residues were pressed free of absorbed alcohol in a screw-press. The combined extracts were filtered and concentrated under reduced pressure. The material that separated from the concentrated alcohol extracts was filtered off and extracted with boiling ethyl ether. The ether-insoluble material

(70 gm.) proved to be mannitol. Its identification will be described later. The ether solution was concentrated *in vacuo*, and 18 gm. of crude sterol were obtained. By means of fractional crystallization from 20 volumes of hot alcohol-benzene (2:1), 13 gm. of ergosterol were isolated, m.p. 159–160°; $[\alpha]_D^{25} = -127.1°$ (CHCl$_3$). Two recrystallizations from the same solvent yielded 10 gm. of snow-white needles (Fraction A). These were dried in a vacuum oven for 1 hour at room temperature and then for 15 minutes at 80°.

M.p. 160–161°; $[\alpha]_D^{21} = -130.7°$ ($a = -7.84°$, $c = 3.0$ in CHCl$_3$, $l = 2$). Three investigators (8–10) report melting points ranging between 160–163° and rotations between $-130°$ and $-135°$.

From the concentrated mother liquors of Fraction A, a further quantity of ergosterol was isolated. This was recrystallized from alcohol-benzene (2:1) to yield Fraction B. The filtrate from the previously mentioned 13 gm. of ergosterol was concentrated to one-half its volume and a small quantity of ergosterol crystallized out. The latter was recrystallized from the mother liquor of Fraction B to give Fraction C. The absorption spectra of Fractions A, B, and C were obtained as will be described later.

A quantity of less pure ergosterol, about 10 gm. (Fraction D), was obtained from the original alcohol and ether filtrates after concentration and saponification with 5 per cent alcoholic potassium hydroxide and shaking out with ethyl ether in the customary manner.

Derivatives of Ergosterol—The acetate was prepared by refluxing 0.3 gm. of Fraction A, 8 cc. of acetic anhydride, and a very small piece of anhydrous zinc chloride in a water bath for 1 hour. After cooling, the solid material was filtered off, washed with 80 per cent acetone, and recrystallized from ether-acetone (1:3).

M.p. 173–175°; $[\alpha]_D^{19} = -90.0°$ ($a = -1.80°$, $c = 1.0$ in CHCl$_3$, $l = 2$). Reindel *et al.* (11) give the melting point as 172–173° and the optical rotation as $-87.4°$.

In preparing the benzoate, 0.9 gm. of Fraction A was dissolved in 18 cc. of pyridine. This solution was cooled and to it were added, drop by drop, 2 cc. of benzoyl chloride. The mixture was allowed to stand at room temperature for 24 hours. It was next diluted with water and shaken out three times with ethyl ether.

The ether extracts were washed with water and then evaporated to dryness. The crystalline residue was recrystallized twice from ethyl acetate.

M.p. 168–169°; $[\alpha]_D^{26} = -67.7°$ ($a = -4.06°$, $c = 3.0$ in $CHCl_3$, $l = 2$). Windaus and Rygh (12) and Wieland and Asano (9) report the melting point as 168° and 168–170°, respectively, and the rotation as $-68°$ and $-71.5°$.

Absorption Spectra—Spectrographic studies were made with a Hilger quartz spectrograph (E-37), sector photometer, hydrogen discharge tube, and Moll recording microphotometer.[1] The absorption spectra of the three ergosterol fractions (Fractions A, B, and C) were obtained by placing in front of the spectrograph slit a quartz absorption cell (2 cm.) containing a 0.0016 per cent solution of the sterol in 96 per cent alcohol. A control exposure was made with alcohol only in the cell. All three fractions showed the characteristic absorption bands of ergosterol, namely at λ 260, 270, 282, and 293.5 $m\mu$ (13). In 0.002 per cent concentration in alcohol, the molecular extinction coefficients of Fraction A were 6700 at λ 260 $m\mu$, 9550 at λ 270 $m\mu$, 10,500 at λ 282 $m\mu$, and 5740 at λ 293.5 $m\mu$.

The mother liquors from Fractions C and D were combined and examined spectrographically. Fig. 1 is the Moll microphotometer tracing of the spectrum obtained. It clearly shows the absorption maxima of ergosterol, as described by Bills *et al.* (13), and also indicates, by the absence of other maxima, that no sterols having an absorption spectrum in this range and at this concentration were contained in the mother liquors.

Identification of Mannitol

Mannitol has long been known to be a constituent of fungous tissues. Raistrick and his coworkers (14) have recently shown that it can also be isolated in good yield (50 per cent of the glucose fermented) from the culture medium of certain species of *Aspergilli*.

The material (70 gm.) that separated from the concentrated

alcohol extracts of the mold mycelium and which was insoluble in ether was identified as mannitol.   10 gm. of the substance were dissolved in 100 cc. of water, the solution was filtered, and the filtrate evaporated on a steam bath to about 30 cc.   This concentrated solution was allowed to crystallize at room temperature for 2 days.   The large, white, needle clusters were filtered off, washed with alcohol and ether, and dried in a vacuum for ½ hour at 60°.
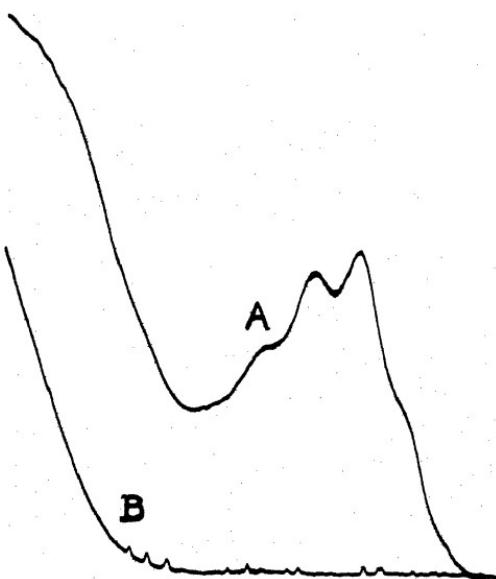
Fig. 1. Moll microphotometer tracing of the ultra-violet absorption spectra of (A) sterol in mother liquors (concentration 0.02 mg. per cc., cell thickness 2 cm.), (B) solvent (ethyl alcohol).

M.p. 166°; $[\alpha]_D^{25} = +27.7°$ ($a = +2.77°$, $c = 5.0$ plus 9 gm. of borax in water, $l = 2$).   These constants agree very well with those reported by Walton and Fort, (15); viz., m.p. 166°; $[\alpha]_D^{20} = +25.9°$.

Hexaacetyl mannitol was prepared by refluxing 1 gm. of the mannitol with 5 cc. of acetic anhydride and a small piece of an-

hydrous zinc chloride. After 1 hour of heating, the mass was poured into water and the solid so produced was filtered off and recrystallized from alcohol.

The acetate melted at 119°. This is identical with the melting point given by Thorpe and Whiteley (16).

The tribenzal derivative was also prepared. 5 gm. of the mannitol, 8 cc. of benzaldehyde, 1 gm. of zinc chloride, and 10 cc. of concentrated hydrochloric acid were well mixed. After standing 24 hours, the solidified mass was transferred to a Buchner funnel, where it was washed with water and alcohol. The derivative was recrystallized from boiling benzene, and dried in a vacuum for ½ hour at 60°. M.p. 218–220°. Walton and Fort (15) report m.p. 220°.

Mannitol and ergosterol have also been isolated from the mycelium of another mold, *Aspergillus oryzæ*, Culture 965. This mold was grown in the large sterilizer-incubators on the inorganic salts-glucose medium, but in addition this medium contained an excess of calcium carbonate.

SUMMARY

Ergosterol and mannitol have been isolated from *Aspergillus fischeri* (Thom 5041) and *Aspergillus oryzæ*, Culture 965, grown on an inorganic salt medium containing 15 per cent glucose as the source of carbon, and have been identified.

The melting points and optical rotations of the free sterol, its acetate and benzoate, as well as the absorption spectra of the sterol have been used to characterize the ergosterol.

Mannitol has been identified by means of its melting point, optical rotation in borax solution, and the melting points of its tribenzal and hexaacetyl derivatives.

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THE RING STRUCTURE OF GUANOSINE

By P. A. LEVENE AND R. STUART TIPSON

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In the case of one of the four known ribosenucleosides, adenosine, the ring structure has been established by direct experimental methods. The present communication deals with the ring structure of guanosine. The general procedure employed in the case of adenosine was followed on the present occasion with satisfactory results. It must be remarked, however, that contrary to expectation, the methylated guanosine was found more resistant to hydrolysis than the methylated adenosine. Methylated adenosine was hydrolyzed without difficulty on heating a solution of it in 4 per cent hydrochloric acid for 2 hours at 85°, whereas for a satisfactory yield of the methylated sugar from methylated guanosine, heating at 85° for 8 hours was necessary.

Guanosine was completely methylated by treatment of a solution of guanosine acetate in acetone with dimethyl sulfate and alkali. The product, trimethyl-N-methylguanosine, was isolated in the form of its hydrochloride, an amorphous powder.

Hydrolysis of the methylated nucleoside gave a sugar the composition of which corresponded to that for trimethyl ribose, the refractive index and specific rotation agreeing closely with those previously recorded[1] for 2,3,5-trimethyl ribose from adenosine.

In order to confirm its identity, the sugar was oxidized with nitric acid under conditions in which trimethyl ribopyranose gives trimethoxyglutaric acid exclusively. In the present instance the sole product was *i*-dimethoxysuccinic acid, which was isolated and identified in the form of its crystalline dimethyl ester.

It follows that guanosine, like adenosine, is a ribofuranoside.

[1] Levene, P. A., and Tipson, R. S., *J. Biol. Chem.*, **94**, 809 (1931–32).

$$
\begin{array}{c}
\text{HN—CO} \\
| \quad\; | \\
\text{H}_2\text{N·C} \quad \text{C————N} \\
\| \quad\;\; \| \quad\quad / \\
\text{N—C—N=CH}
\end{array}
$$

$$
\begin{array}{c}
\text{HN—CO} \\
| \quad\; | \\
\text{HCl·HN—C} \quad \text{C————N} \\
/ \quad\quad \| \quad\;\; \| \quad\quad / \\
\text{CH}_3 \quad\; \text{N—C—N=CH}
\end{array}
$$

$\longrightarrow$

$$
\begin{array}{c}
\text{H·C———} \\
| \quad\quad\;| \\
\text{H·C·OH} \;\; | \\
| \quad\quad\;\; \text{O} \\
\text{H·C·OH} \;\; | \\
| \quad\quad\;| \\
\text{H·C————} \\
| \\
\text{CH}_2\text{OH}
\end{array}
$$

Guanosine

$$
\begin{array}{c}
\text{H·C———} \\
| \quad\quad\;\;| \\
\text{H·C·OMe} \;\; | \\
| \quad\quad\quad \text{O} \\
\text{H·C·OMe} \;\; | \\
| \quad\quad\;\;| \\
\text{H·C————} \\
| \\
\text{CH}_2\text{OMe}
\end{array}
$$

Trimethyl-N-methylguanosine
hydrochloride

$\longrightarrow$

$$
\begin{array}{c}
\text{CHOH———} \\
| \quad\quad\;\;| \\
\text{H·C·OMe} \;\; | \\
| \quad\quad\quad \text{O} \\
\text{H·C·OMe} \;\; | \\
| \quad\quad\;\;| \\
\text{H·C————} \\
| \\
\text{CH}_2\text{OMe}
\end{array}
$$

2,3,5-Trimethyl ribose

$\longrightarrow$

$$
\begin{array}{c}
\text{COOH} \\
| \\
\text{H·C·OMe} \\
| \\
\text{H·C·OMe} \\
| \\
\text{COOH}
\end{array}
$$

i-Dimethoxysuccinic acid
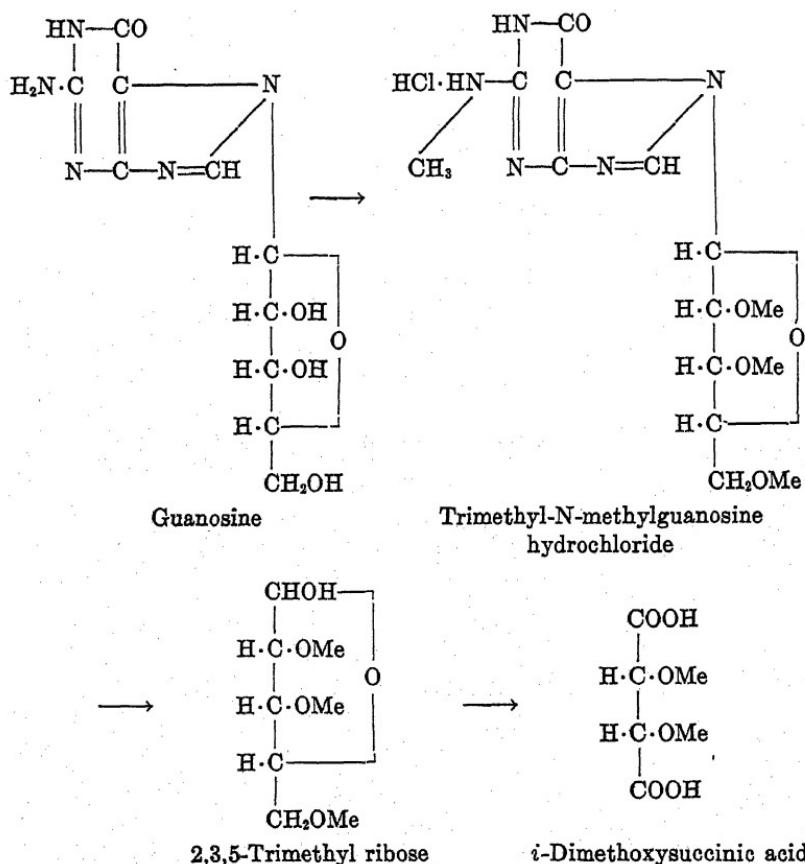
EXPERIMENTAL

Preparation of Triacetyl Guanosine[2]—A suspension of 0.25 gm. of fused sodium acetate in 130 cc. of acetic anhydride was heated to gentle ebullition and then finely powdered guanosine hydrate (10 gm.) was added in portions sufficient to keep the mixture boiling. When all the guanosine had been added, the resultant clear pale yellow solution was evaporated to a thick gum under diminished pressure. The product was dissolved in 300 cc. of acetone, the trace of undissolved sodium acetate filtered off, and the filtrate evaporated to dryness under diminished pressure. The guanosine acetate was obtained as a pale yellow, flaky, glass-like solid.

[2] Steudel, H., and Friese, R., *Z. physiol. Chem.*, **120**, 126 (1922).

Yield, 12 to 12.5 gm.   It was insoluble in cold or hot carbon tetra-chloride, petroleum ether, ether, or toluene; but soluble in the following solvents in the cold: chloroform, glacial acetic acid, pyridine, acetone, methyl alcohol, ethyl alcohol, ethyl acetate, and water.   It was obtained crystalline by allowing a fairly concentrated solution of the glassy acetate in chloroform-petroleum ether to stand in the refrigerator.

The colorless crystalline material had a melting point of 224–225° (uncorrected).

4.285 mg. substance: 7.370 mg. CO_2 and 1.725 mg. H_2O
5.669 "   "   : 0.840 cc. N_2 (758 mm. at 24°) (Dumas)
22.56 "   "   : 1.42 " " (764 "   " 22°) (Van Slyke)
$C_{16}H_{19}O_8N_5$. Calculated. C 46.92, H 4.68, N 17.11, amino N 3.42
           Found.   " 46.91, " 4.50, " 16.99, "   " 3.56

Methylation of Guanosine—The acetate from 10 gm. of guanosine hydrate was dissolved in 300 cc. of acetone and treated with 228 cc. of 30 per cent sodium hydroxide solution and 112 cc. of dimethyl sulfate as previously described for the methylation of adenosine.[1]

The product was a pale brown, glassy solid, soluble in acetone, methyl alcohol, and water; insoluble in ether or petroleum ether. Yield, 9.6 gm.   It was dissolved in 35 cc. of absolute methyl alcohol, 15 cc. of dry ether were added, and the solution was cooled to 0° in a freezing mixture.   A slow stream of dry hydrogen chloride was now passed into the solution to slight excess.   No crystalline material separated, so the solution was evaporated at room temperature to a gum which was readily obtained as a fine powder by grinding under a layer of dry ether in a mortar.   The powdery hydrochloride, which decomposed at 98°, was dried at 80° for analysis.

3.896 mg. substance: 6.430 mg. CO_2 and 2.180 mg. H_2O
4.904 "   "   : 0.774 cc. N_2 (765 mm. at 24°)
3.716 "   "   : 6.050 mg. AgI
$C_{14}H_{22}O_5N_5Cl$. Calculated. C 44.72, H 5.9, N 18.64, OMe 24.8
           Found.   " 45.00, " 6.3, " 18.26, "   21.5

Hydrolysis of Trimethyl-N-Methylguanosine—In preliminary experiments the hydrolysis was conducted as previously described for trimethyl-N-methyladenosine[1] but the yield of methylated

sugar amounted to only 25 per cent of the theoretical, presumably owing to the stability of the methylated nucleoside.   In a typical experiment, 6 gm. of the powdery hydrochloride were dissolved in 120 cc. of 4 per cent hydrochloric acid and the resultant solution was heated under a reflux condenser in a bath at 85° for 2 hours. The hot solution was then made neutral by addition of barium carbonate, heated until boiling to expel excess carbon dioxide, and then kept in the refrigerator overnight.   No base had separated, so the solution was extracted several times with chloroform and then evaporated to dryness and the barium chloride extracted with boiling chloroform under a reflux.   The united chloroform extracts were dried over anhydrous sodium sulfate, filtered, and the filtrate evaporated to dryness under diminished pressure.   The resulting pale brown syrup was not completely soluble in dry ether, owing to the presence of unhydrolyzed nucleoside.   The ether was filtered, and the filtrate evaporated under diminished pressure to a pale yellow syrup which was strongly reducing to boiling Fehling's solution.   The product was distilled at high vacuum at 80–85° at 0.03 mm. (bath temperature 110–115°).   Yield 0.76 gm. $n_D^{24} =$ 1.4548.   The substance, a colorless mobile syrup, had the following composition:

4.513 mg. substance: 8.221 mg. CO_2 and 3.420 mg. H_2O
3.600 "   "   : 12.600 " AgI
$\qquad$ $C_8H_{16}O_5$.   Calculated.   C 49.97, H 8.4, OMe 48.45
$\qquad\qquad$ Found.   " 49.72, " 8.5, "   46.24

The syrupy distilled product had the following specific rotation.

$$[\alpha]_D^{25} = \frac{+0.91° \times 100}{2 \times 1.100} = +41.4° \text{ (in absolute methyl alcohol)}$$

In another experiment, in which the period of hydrolysis was extended to 8 hours, 11.1 gm. of trimethyl-N-methylguanosine gave 4.1 gm. of trimethyl ribose (66 per cent of the theoretical).

Oxidation of Trimethyl Ribose with Nitric Acid—Syrupy distilled trimethyl ribose (9 gm.) was dissolved in 90 cc. of concentrated nitric acid (specific gravity 1.42) at 26°.   The solution was heated in a bath at 95° during 7 hours, after which gas evolution had ceased.   The reaction mixture was cooled, diluted with distilled water, and nitric acid removed under diminished pressure in

the usual manner.[1]   The dried product was esterified by boiling for 9 hours with 200 cc. of methyl alcohol containing 2 per cent of hydrogen chloride.   The ester was purified by distillation at high vacuum.   A colorless syrupy product was collected at 74° at 0.06 mm. (bath temperature 90–92°).   Yield, 3.4 gm. $n_D^{26} =$ 1.4343.   It crystallized immediately to a solid mass on nucleation with an authentic specimen of dimethyl i-dimethoxysuccinate. It was recrystallized from ether-light petroleum ether, being obtained in flat plates (melting point 68° alone or admixed with an authentic specimen of dimethyl i-dimethoxysuccinate prepared from mesotartaric acid).

The substance had the following composition:

4.065 mg. substance: 6.915 mg. CO_2 and 2.460 mg. H_2O
4.463 "   "   : 20.346 " AgI
          $C_8H_{14}O_6$.   Calculated.   C 46.58, H 6.8, OMe 60.21
                    Found.   " 46.39, " 6.8, "   60.17

It was optically inactive under conditions ($c = 1.0$; $l = 2$ dm.) where a specific rotation of $\pm 1°$ would easily have been detectable.

The melting point, optical inactivity, and analytical figures showed it to be the dimethyl ester of inactive dimethoxysuccinic acid.
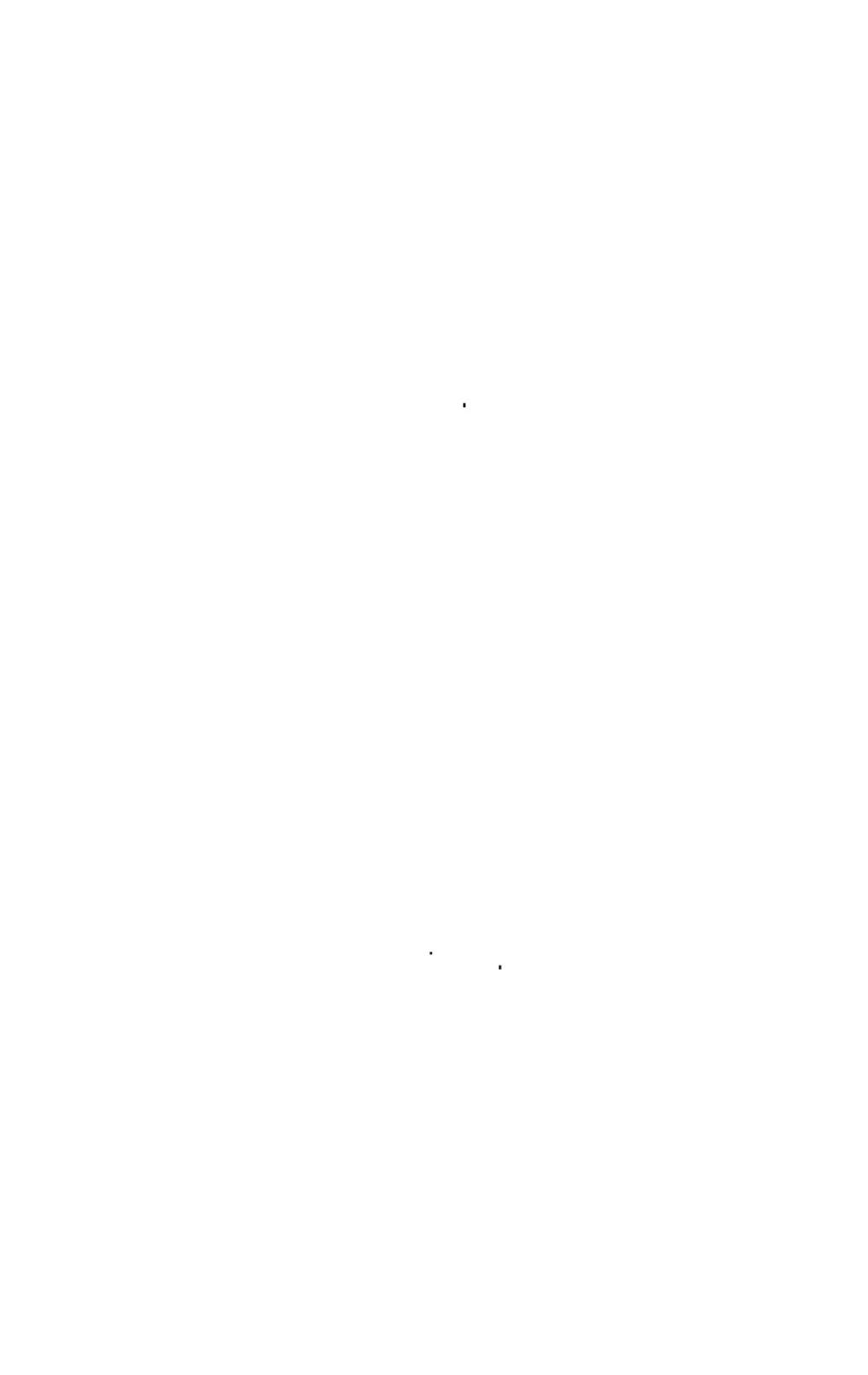
THE EFFECT OF PHLORHIZIN ON THE RATE OF ABSORPTION FROM THE GASTROINTESTINAL TRACT OF THE WHITE RAT

By ROBERT H. WILSON*

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In a study of the rate of absorption of amino acids, Wilson and Lewis (1) found that glycine and alanine were absorbed from the intestinal tract of the white rat much more slowly than Cori (2) had found for glucose. However, Csonka (3) fed isoglucogenic quantities of glycine, alanine, and glucose to phlorhizinized dogs and concluded that "the rapidity of the absorption and elimination of glucose ingested in phlorhizin glycosuria is almost the same as the rapidity of the absorption, deamination, synthetic sugar production, and the elimination of such sugar, after ingestion of iso-glucogenic quantities of glycocoll or alanine." Wilson and Lewis pointed out that "If glucose was excreted by these animals as soon as it entered the blood, the conclusion could be drawn that the time required for the absorption of the amino acids and the sugar formation from them must have been of the same order as the time required for the absorption of the glucose." In an attempt to explain the apparent lack of agreement between these two sets of results, several possible reasons were mentioned: there might be a period of storage of glucose in the body after its absorption; variation in the rate of absorption might be due to difference in species; the rapid excretion of glucose after the amino acid ingestion in Csonka's experiment might be caused by a stimulation of excretion of preexisting glucose; or finally, phlorhizinization might so alter the wall of the intestine as to make it more permeable to the amino acids, less permeable to glucose, or a combination of these two actions. Theoretical or indirect arguments were found

against the first three of these possibilities. An investigation of the action of phlorhizin on intestinal permeability is given in this paper.

Nakazawa (4) studied the change in rate of absorption in the intestine when phlorhizin was introduced into the lumen of the gut. Two equally long, adjacent segments of intestine were formed by ligation of the intestine of a narcotized rabbit. The substance to be studied was placed in one segment and an equal amount with some phlorhizin in the other. At the end of the experimental period the amount remaining in each section was determined. Under these conditions the absorption of glucose was strongly repressed by the phlorhizin, while there was no change in the rate of absorption of sodium chloride, glycine, or fatty acids. Indicative as these results are of a change in the rate of intestinal absorption due to the presence of phlorhizin, they cannot be applied directly to such experiments as those of Csonka. The anesthesia, the operative procedure, and the introduction of the drug directly into the gut instead of indirectly by way of the systemic circulation, might modify the action.

In the present study the Cori (2) method was used to determine the rate of absorption. The intestinal washings were treated with tungstic acid to remove protein. Glucose was estimated by the Hagedorn-Jensen method and amino acids by the Van Slyke procedure. The rats were phlorhizinized by four daily subcutaneous injections of 50 mg. of phlorhizin suspended in olive oil (5). Food was withheld from the animals for the 24 hours before the absorption was to be tested.

The residual reducing substances and residual amino nitrogen in the intestine after a 24 hour fast were found to be unchanged by the phlorhizin injections. The rate of absorption of glucose (Table I) in the normal animal as found in this experiment (201 mg. per 100 gm. per hour) agrees remarkably closely with the value obtained by Cori. Phlorhizin decreased this rate to about 70 per cent of the normal, a finding in agreement with that of Nakazawa. However, the statement of Nakazawa that the rate of glycine absorption was not affected by phlorhizin could not be confirmed. In the experiments with glycine (Table II) and alanine (Table III) there was a distinctly greater rate of absorption by phlorhi-

zinized animals than was found for normal rats. The values obtained for the normal animals agree very well with those reported by Wilson and Lewis if the 3 hour absorption period is considered. The normal values for a 2 hour absorption period run considerably higher than was previously found.

TABLE I

Rate of Absorption of Glucose in Normal and Phlorhizinized Rats

			Normal rats						Phlorhizinized rats		
			Glucose						Glucose		
Rat No.	Weight after fast	Absorption time	Fed	Recovered	Rate of absorption	Rat No.	Weight after fast	Absorption time	Fed	Recovered	Rate of absorption
	gm.	*hrs.*	*mg. per 100 gm.*	*mg. per 100 gm.*	*mg. per 100 gm. per hr.*		*gm.*	*hrs.*	*mg. per 100 gm.*	*mg. per 100 gm.*	*mg. per 100 gm. per hr.*
58	124	2	734	325	205	66	179	2	488	218	135
59	113	2	805	356	225	68	167	2	523	169	177
62	130	2	703	230	237	70	157	2	573	252	161
64	165	2	554	185	185	71	176	2	511	194	159
						75	196	2	594	426	84
						76	199	2	585	409	88
Average.................					213 ±14	Average.................					134 ±24
57	134	3	679	109	190	65	176	3	496	62	145
60	125	3	728	158	190	67	150	3	583	129	151
61	118	3	774	283	164	69	154	3	585	278	102
63	127	3	720	75	215	72	168	3	536	137	133
						73	164	3	710	133	192
Average.................					190 ±10	Average.................					145 ±16
Average of all experiments.................					201 ±14	Average of all phlorhizin experiments....					139 ±20

All computations were made on the basis of the weight of the animal after the 24 hour fast. Since the phlorhizinized animals lost about 5 per cent more of their original weight than did the untreated rats, the values are not strictly comparable. However, this slightly greater weight loss is not large enough to account for the increased rate of absorption of the amino acids, and it would

cause a corresponding decrease of glucose absorption to even lower values.

The equal rates of excretion of glucose following the ingestion of glucose, glycine, and alanine by phlorhizinized dogs is partially explained by this demonstration of a decreased rate of absorption

TABLE II

Rate of Absorption of Glycine in Normal and Phlorhizinized Rats

			Normal rats						Phlorhizinized rats		
			Glycine						Glycine		
Rat No.	Weight after fast	Absorption time	Fed	Recovered	Rate of absorption	Rat No.	Weight after fast	Absorption time	Fed	Recovered	Rate of absorption
	gm.	*hrs.*	*mg. per 100 gm.*	*mg. per 100 gm.*	*mg. per 100 gm. per hr.*		*gm.*	*hrs.*	*mg. per 100 gm.*	*mg. per 100 gm.*	*mg. per 100 gm. per hr.*
90	149	2	175	25	73	103	174	2	181	10	86
92	130	2	200	47	77	104	170	2	185	26	80
93	146	2	195	71	62	105	126	2	293	118	88
95	156	2	182	59	62	106	126	2	293	83	105
138	134	2	260	104	78	109	165	2	218	94	62
139	121	2	288	144	72	110	153	2	234	51	92
Average................					71 ±4	Average................					86 ±7
89	164	3	159	14	48	101	110	3	286	122	55
91	139	3	188	38	50	102	146	3	216	0	72
94	145	3	196	48	49	107	100	3	369	40	110
96	145	3	196	52	48	108	121	3	305	42	88
						112	106	3	339	66	91
Average................					49 ±1	Average................					83 ±12
Average of all experiments................					62 ±8	Average of all phlorhizin experiments....					85 ± 9

of glucose and an increased rate for glycine and alanine in phlorhizin glycosuria in rats. The explanation is not complete, however. As computed on the basis of maximum glucose formation, alanine is absorbed by phlorhizinized rats at 84 per cent of the rate for glucose, while the rate for glycine is only 49 per cent. Some other

factor must enter into the explanation of the equal excretory rates found by Csonka.

TABLE III

Rate of Absorption of Alanine in Normal and Phlorhizinized Rats

			Normal rats						Phlorhizinized rats		
			Alanine						Alanine		
Rat No.	Weight after fast	Absorption time	Fed	Recovered	Rate of absorption	Rat No.	Weight after fast	Absorption time	Fed	Recovered	Rate of absorption
	gm.	hrs.	mg. per 100 gm.	mg. per 100 gm.	mg. per 100 gm. per hr.		gm.	hrs.	mg. per 100 gm.	mg. per 100 gm.	mg. per 100 gm. per hr.
119	176	2	169	8	80	125	121	2	272	30	121
120	172	2	173	43	70	128	118	2	279	40	120
123	129	2	298	72	113	129	117	2	326	38	144
124	178	2	216	16	100	130	102	2	374	116	129
136	130	2	292	65	114	132	128	2	294	106	94
137	145	2	262	85	89	133	121	2	310	45	133
Average.................					94 ±11	Average.................					124 ±9
117	108	3	275	0	92	126	102	3	374	36	113
118	160	3	186	0	62	127	116	3	284	10	91
121	124	3	310	66	81	131	105	3	363	10	118
122	123	3	312	119	64	134	114	3	330	0	110
						135	119	3	316	25	97
Average.................					75 ± 9	Average.................					106 ±7
Average of all experiments.................					87 ±11	Average of all phlorhizin experiments....					116 ±9

SUMMARY

White rats receiving four daily subcutaneous injections of phlorhizin were found to absorb glucose at 70 per cent of the normal rate, while the rate was increased for glycine and alanine to 137 per cent and 134 per cent of the normals, respectively. The changed rates of absorption due to the drug furnish a partial explanation of the equal rates of excretion of glucose in phlorhizin glycosuria of dogs after the ingestion of these three substances.

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QUINCE SEED MUCILAGE

By ALICE G. RENFREW and LEONARD H. CRETCHER

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Recent studies of plant gums and plant tissues have resulted in
the identification of an increasing number of polysaccharide-uronic
acid complexes. On hydrolysis the polysaccharides often yield
several different sugars as well as an aldobionic acid unit. Usually
the aldobionic acid consists of a hexose sugar in combination with
a uronic acid, although aldobionic acids containing pentose have
been found. To date, glycuronic and galacturonic acids are the
only uronic acids which have been identified in these groupings.
The only recognized variation has been that in some cases the
uronic acids occur in methylated form. Methoxyglycuronic acid
has been found by Anderson and Otis (1) in mesquite gum. Methyl-
ated uronic acids have also been found in cherry gum by Butler
and Cretcher (2) and in the gum from lemon trees by Anderson
and Russel (3).

Quince seed mucilage affords an additional example of the
natural occurrence of methoxyuronic acids. As was suggested
by Schmidt (4) and more definitely shown by Kirchner and Tol-
lens (5), quince seed mucilage is a complex of a cellulosic fraction
with a more readily hydrolyzed polysaccharide. The linkage is
not easily broken by the action of dilute acid or alkali at room tem-
perature, but hot dilute acid liberates reducing sugars, cellulose,
and "a gum" (5). In the present investigation the decomposition
products found after hydrolysis with 0.5 N sulfuric acid were in-
organic salts, about 33 per cent of the cellulosic residue, a small
amount of arabinose, and a mixture of aldobionic acids—a mix-
ture composed of methylated and unmethylated hexuronic acids in
combination with xylose. The simplest interpretation of the
Zeisel analysis would indicate the presence of 72 per cent of a

503

monomethylated and 28 per cent of an unmethylated hexuronic acid.

The occurrence of xylose-uronic acid complexes has already been reported for several gums of hemicellulosic origin. Anderson and Kinsman (6) have found a xylose-glycuronic acid unit in the hemicellulose of cottonseed hulls; Sands and Gary (7) found xylose linked with uronic acid in the hemicellulose of mesquite wood; and Preece (8) isolated a xylose-uronic acid complex from boxwood.

<div align="center">EXPERIMENTAL</div>

Preparation of Quince Seed Gum—The quince seeds used were classified as *Cydonia vulgaris* and were of Persian origin. For the preparation of the gum the seeds were first extracted with alcohol and with ether, or with 50 per cent alcohol-ether. Subsequently 500 gm. were treated with 3 liters and then with 1 liter of water, during a total period of 24 hours. The resultant mucilage was drained from the seeds on a Buchner funnel and filtered through cloth. The gum was precipitated with several volumes of alcohol, dehydrated with alcohol and acetone, and pressed in a hydraulic press. The neutral gum is readily soluble in water and contains about 6 per cent ash, 25 per cent SiO_2, 15 per cent R_2O_3, 16 per cent CaO, and 8 per cent MgO, with a considerable amount of phosphate. The ash also gives a strong flame test for potassium.

To obtain a gum with less ash, precipitation was carried out in a solution containing about 1 per cent hydrochloric acid. The coagulum was collected, dissolved in dilute alkali, and again precipitated from a slightly acid solution. The dehydrated acidic gum was no longer soluble in water except with the addition of dilute alkali. The carefully washed gum had a neutralization equivalent corresponding to the uronic acid content as estimated by the Lefèvre carbon dioxide method (9). When the gum was prepared by precipitation in an acidified solution, it was possible to separate arabinose and a more soluble gum fraction from the concentrated liquors; this more soluble gum contained about 30 per cent uronic acid and 52 per cent pentose. No study was made of the supernatant liquor from the neutral gum.

While the conversion of uronic acid to CO_2 is ordinarily considered quantitative, the occurrence of a methoxyuronic acid introduces an undetermined factor in relation to the yield of CO_2

and of furfural.   Moreover, the lack of quantitative methods for the estimation of xylose makes impossible a countercheck against the furfural determinations.   In the absence of more precise knowledge, it was assumed that the conversion of methoxyuronic acid to CO_2 is quantitative, and the CO_2 value was regarded as 21.1 per cent of the weight of the methylated uronic acid.   In all analyses the sample was heated with 2 per cent hydrochloric acid to decompose any carbonates.   The standard values for the yield of furfural from uronic acid were used (10).

TABLE I

Analytical Values for Quince Gum

	Acidic gum	Neutral gum
	per cent	*per cent*
CH_3O...	3.3	2.9
CHO (iodine titration)........................	1.4	
Uronic acid (titration with 0.1 N NaOH).......	26.1	
CO_2...	6.0	5.6
Uronic acid (calculated from CO_2)...........	27.8	26.0
Pentose (furfural corrected for uronic acid)....	33.0	31.0
Ash...	2.0	6.0
Cellulose.....................................	33.0	33.0
Total.....................................	95.8	96.0

Elementary analysis: C 42.43, H 6.25.

A Zeisel determination on the neutral gum gave a methoxy content of 2.9 per cent.   There was no ready removal of the methoxyl group from the gum or from the aldobionic acid by dilute alkali under the conditions described by Ehrlich (11) for the hydrolysis of methyl esters found in pectin.   The neutral gum gave no test for the presence of acetyl groups.   Both of these tests were made as a check against the possible presence of appreciable amounts of pectin from the fruit pulp.

Table I shows the analytical results.

Hydrolysis of Quince Gum with 0.5 N H_2SO_4—The quince gum was allowed to stand some hours in 30 times its weight of water. After the addition of sulfuric acid to a concentration of 0.5 N, the solution was refluxed for about 10 hours until little further

increase in optical rotation was observed. For a solution having an initial observed angle of $\alpha = +0.50°$, the observed angle reached the value of $+1.64°$ in 12 hours.

At the end of the hydrolysis there remained a bulky residue representing about one-third of the dry weight of the gum. Kirchner and Tollens (5) reported 34 per cent of a cellulosic residue, about half of which was soluble in Schweitzer's reagent. The residue obtained in the present investigation had an elementary composition corresponding closely to $C_6H_{10}O_5$. Calculated, C 44.47, H 6.17. Found, C 43.78, H 6.44. About 28 per cent of the residue was removed as β- and γ-cellulose by the action of 17.5 per cent NaOH; most of this was reprecipitated by acetic acid. The residual α-cellulose was hydrolyzed by the method of Monier-Williams (12) with a 78 per cent yield of crystalline glucose. The glucose was identified by rotation and by the preparation of glucosazone.

Hydrolysis of the gum at room temperature with 75 per cent sulfuric acid for 5 days left, after dilution, 41 per cent of a black residue. A calcium salt of the aldobionic acid and a crystalline, acetone-soluble osazone with a melting point of about 160° were prepared from the supernatant solution. The yields were lower than those obtained on hydrolysis with dilute acid.

Barium and Calcium Salts of the Aldobionic Acid Fraction—The hydrolysate obtained with 0.5 N sulfuric acid was neutralized with calcium carbonate or with barium hydroxide and barium carbonate. After the removal of sulfate, the salt of an aldobionic acid was precipitated by the addition of alcohol to the concentrated aqueous solution. Analyses of either the barium salts or the calcium salts corresponded quite closely to the values for an aldobionic acid containing 1 mol of a hexuronic acid in combination with 1 mol of pentose. However, the methoxy values for both salts indicated about 72 per cent of a monomethylated aldobionic acid admixed with 28 per cent of a similar unmethylated acid. Before analysis the salts were repeatedly reprecipitated, with the use of methyl or ethyl alcohol. Hence, it would seem that the salts represented a mixture of rather constant composition and that the separation of methylated and unmethylated acids would require a large amount of material.

An aqueous solution of the calcium salt had a specific rotation

of $[\alpha]_D = +63°$; the acidified solution had an initial rotation of $[\alpha]_D = +64°$ (calculated on the weight of calcium-free material) and showed no mutarotation in 24 hours. The analytical values shown in Table II were obtained.

1 gm. of the aldobionic acid (obtained by treating the calcium salt with the calculated amount of oxalic acid) was oxidized with nitric acid under standard conditions for determining mucic acid. No mucic acid was obtained even after seeding the solution, and no potassium acid saccharate was identified. This failure to identify either mucic acid or potassium acid saccharate in any of

TABLE II

Analysis of Barium Salt

Mol. wt. = 807.   72 per cent $(CH_3O \cdot C_{11}H_{16}O_{10})_2 Ba$
28 "   " $(C_{11}H_{17}O_{11})_2 Ba$

	Calculated	Found
	per cent	*per cent*
CH_3O	5.5	5.5
CHO	7.2	7.8
CO_2	10.88	10.7
Uronic acid	50.7	49.7
Pentose*	37	32
Ba	16.8	17.1

* 0.2076 gm. of Ba salt gave 0.0950 gm. of furfuralphloroglucide. On the basis of the CO_2 determination this sample contained 0.1038 gm. of uronic acid, which would give 0.0346 gm. of furfuralphloroglucide (10). The corrected weight of furfuralphloroglucide was 0.0604 gm., or the equivalent of 0.066 gm. of pentose.

the nitric acid oxidations of the whole gum or various fractions of the gum is similar to the experience of other investigators (4, 13, 14).

Identification of l-Arabinose in Hydrolysis Mother Liquors— The supernatant liquors from the precipitation of the aldobionic acid salts were concentrated to dryness, and the dry residue extracted with 95 per cent alcohol. The alcohol-soluble material gave a negative Seliwanoff reaction for ketoses. Diphenylhydrazine was used for the quantitative precipitation of arabinose from an aliquot portion of the alcoholic extracts. The yield of arabinose determined in this manner was 2 per cent of the dry weight of the

gum. The benzylphenylhydrazone melted at 172° and caused no lowering of the melting point when mixed with a known sample of *l*-arabinose benzylphenylhydrazone (15). The specific rotation of this hydrazone was $[\alpha]_D^{25} = -15.9°$ in a 50 per cent methyl alcohol-pyridine solution.

A further yield of low melting hydrazone was obtained by diluting the filtrates; however, only the arabinose hydrazone could be identified in the recrystallized product. No galactose was identified in this manner and no mucic acid could be identified in the nitric acid oxidations.

Various furfuralphloroglucide precipitates were examined for the presence of alcohol-soluble methylfurfural compounds, but these were not found.

Hydrolysis of Aldobionic Acid—Xylose and the Calcium Salt of Mixed Hexuronic Acids—The aldobionic acid is resistant to acid hydrolysis; from a solution refluxed 5 hours only unchanged aldobionic acid was recovered. 14 gm. of the calcium salt were refluxed 20 hours with 600 cc. of 0.96 N sulfuric acid. The solution discolored rapidly and the carbon dioxide evolved corresponded to the destruction of about 21 per cent of the uronic acid. After 3 hours $[\alpha]$ was about $+60°$; the final rotation was much lower. Sulfuric acid was removed as calcium sulfate and the concentrated solution treated with 2 and with 6 volumes of alcohol. About 2.3 gm. of unhydrolyzed aldobionic acid were recovered in this manner.

The supernatant liquors were concentrated to dryness. Much of the dry residue was insoluble in the hot 95 per cent alcohol used to remove reducing sugars. The alcohol-insoluble material proved to be largely a calcium salt soluble in 75 to 80 per cent alcohol. After several reprecipitations this salt gave analytical values which check closely with the calculated values for a hexuronic acid mixture, containing 60 per cent of a monomethylated and 40 per cent of an unmethylated uronic acid (Table III).

After 5 minutes, the specific rotation of an acidified solution of the calcium salt was $[\alpha]_D = +48°$ (calculated on a calcium-free basis). The solution showed no appreciable mutarotation in 24 hours.

In an oxidation of 0.21 gm. of calcium-free material with nitric acid (sp. gr. = 1.15), no mucic acid separated and no potassium acid saccharate could be identified.

The alcoholic solution which should contain the reducing sugars was tested for the presence of arabinose and xylose. No arabinose benzylphenylhydrazone crystallized under standard conditions (10) or when the solution was diluted. Crystalline xylose was isolated from the concentrated alcoholic solution. An 8 per cent solution of the crystalline material had a specific rotation of $[\alpha]_D = +68°$ after 5 minutes. After 2 hours the rotation was $[\alpha]_D = +19.1°$ and remained at this value for 24 hours. The specific rotation for xylose is $[\alpha]_D = +19°$ (16). Xylose was further identified by oxidation of the crystalline material to the characteristic boat-shaped crystals of the double salt of cadmium bromide-cadmium xylonate. Xylose has been previously identified in the hydrolysate of the whole gum by Schulze and Tollens (13) by

TABLE III

Analysis of Calcium Salt of Mixed Uronic Acids

Mol. wt. = 442.   60 per cent $(CH_3O \cdot C_6H_8O_6)_2 Ca$
40   "   " $(C_6H_9O_7)_2 Ca$

	Calculated	Found
	per cent	*per cent*
CH_3O	8.53	8.53
CHO	13.2	13.8
CO_2	20.0	20.7
Ca	9.09	9.33

the isolation of a small amount of the crystalline sugar, which had the specific rotation of xylose. Gans and Tollens (14) isolated a pentose osazone; this latter may have been a derivative of xylose, or of arabinose, as was first assumed by these authors.

SUMMARY

Arabinose, a mixture of methylated and unmethylated aldobionic acids, and a cellulosic fraction were liberated in the hydrolysis of quince seed gum. Xylose was identified in the further hydrolysis of the aldobionic acids.

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THE PHOSPHORUS PARTITION IN THE BLOOD OF RACHITIC AND NON-RACHITIC CALVES*

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During the past decade considerable emphasis has been placed on the relation of blood phosphorus to such physiological disturbances as rickets and osteoporosis. Most of the investigations have been limited to the inorganic phosphorus fraction of the blood serum.

In 1921 Howland and Kramer (1) showed that the inorganic phosphorus of the blood serum was distinctly lower in children suffering from rickets than in normal infants. Similar results were obtained by von Meysenbug (2), György (3), and Hess and Lundagen (4). A definite decrease was also observed in the blood of rachitic rats by Kramer and Howland (5) and Gutman and Franz (6). Steenbock, Hart, Jones, and Black (7) in their studies on the antirachitic vitamin used the inorganic phosphorus content of the blood serum as one means of diagnosing rickets in such experimental animals as dogs and chickens. Practically all workers who have studied rickets have given some attention to the serum phosphate of blood.

Hess and Unger (8), however, have always been of the opinion that while a decrease in the inorganic phosphorus is generally the case in rickets, it is not specific for the disease. In a recent paper Hess and his collaborators (9) present results which lead them to believe that rickets may be produced in rats, and then the phosphate content of the blood raised to normal by the addition of

small amounts of irradiated ergosterol without signs of healing in the rachitic lesions.

It has been the common practice in many laboratories to allow the blood to clot and then draw off the serum for phosphorus analysis. The blood samples therefore generally stand 24 hours before the determinations are made, and often some hemolysis occurs during this delay. Since Kay and Robison (10) have shown that there is present in blood an acid-soluble organic phosphorus compound which is rapidly hydrolyzed by the enzyme of ossifying cartilage, and since Kay (11) has shown that certain bone diseases may cause a considerable increase in plasma phosphatase, we felt that many of the high inorganic phosphate values observed for rachitic blood might be due to hydrolysis taking place during the interval between the time the sample is taken and the analysis is made. Martland and Robison (12) have called attention to the fact that inorganic phosphorus in blood increases very rapidly after laking. A slight hemolysis would therefore tend to increase the inorganic phosphate values, especially when the blood contains extra amounts of phosphatase. Kramer, Shear, and Siegel (13) have suggested that the negative results reported by Hess et al. (9) may be dependent to some extent upon higher inorganic serum phosphate values obtained in hemolyzed sera.

We were interested in studying the amount of the various phosphorus fractions in normal and rachitic blood when special precautions were taken to make the determinations immediately after the blood was drawn. We chose calves as our experimental animals for two reasons; first, because samples of blood large enough to study all the phosphorus constituents can be obtained from these animals, and secondly, because studies on the vitamin D requirement of calves being carried out by Rupel, Bohstedt, and Hart at this Station made available several normal and rachitic calves.

Data on the phosphorus partition in beef blood are rather meager. Hart and coworkers (14) and Meigs, Blatherwick, and Cary (15) made a few analyses on the blood of dairy cows used in calcium metabolism studies. Robinson and Huffman (16) report inorganic phosphorus values of 3.00 to 8.99 mg. per 100 cc. of plasma from normal beef blood. Palmer, Cunningham, and

Eckles (17) have given careful consideration to the factors which affect the inorganic phosphate content of the serum from the blood of dairy cattle. Hayden and Fish (18) and Anderson, Gayley, and Pratt (19) have studied the acid-soluble phosphorus, as well as the inorganic phosphate, in the serum of normal bovine blood. This work, like most of that conducted with other animals, has been limited to the phosphorus in the serum or plasma. Since the plasma contains less than one-half of the total phosphorus in blood, it seems logical that the rest of the phosphorus should be given attention.

Malan and Green and Macaskill (20) are the only workers known to us who have studied all of the phosphorus in beef blood. Their work dealt mainly with the cattle pasturing on mineral-deficient areas of South Africa. They also observed the phosphorus partition in the blood of calves, from the fetus stage through various periods of growth, and of anemic cattle and sheep. The purpose of this paper is to study the phosphorus fractions in the whole blood of calves on standardized rations and to determine the effect of rickets on these fractions.

EXPERIMENTAL

The animals studied were those used by Rupel, Bohstedt, and Hart in an experiment on the vitamin D requirement of calves. The details of this experiment will be published by them in another paper.[1] It is sufficient to say that twenty-four Holstein calves were used in the experiment and that they were divided into six groups. All calves received the same basal rachitogenic ration, but certain groups were given vitamin supplements. The additions were made as follows:

Group 1, basal ration
2,   plus cod liver oil
3,   aerated cod liver oil
4,   sunlight
5,   tomatoes plus sunlight
6,

The calves were bled when they were 8½ to 9 months old. At this time the animals in Lots 1 and 6 were definitely rachitic

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[1] A preliminary report of this work was given before the American Society of Animal Production at Chicago, January, 1932.

while those in Lots 2 to 5 were in good physical condition.   The blood was taken from the jugular vein and oxalated to prevent clotting.   The samples were brought directly to the laboratory for analysis.   The trichloroacetic acid filtrates and the lecithin filtrates were made and the samples for total phosphorus were dried within an hour after the blood was obtained from the animals.

The technique used in this study was very similar to that outlined by Walker and Huntsinger (21) in their study of the phosphorus partition in normal, whole, human blood.   However, the method of wet combustion for total phosphorus was found very inconvenient and in its place a dry combustion, with $MgNO_3$, in an electric furnace was used.   All the phosphorus determinations were made by the Fiske and Subbarow (22) method.

The reagents were prepared as outlined by Walker and Huntsinger.   The following procedures were used for the preparation of the different fractions.

Trichloroacetic Acid Filtrate—20 cc. of the trichloroacetic acid were pipetted into a 50 cc. Erlenmeyer flask.   5 cc. of whole blood were added slowly from an Ostwald pipette.   The flask was stoppered and the contents shaken vigorously for about a minute.   After 15 minutes the material was filtered, and the filtrate served for the determination of inorganic phosphorus and of acid-soluble phosphorus.

Inorganic Phosphorus—5 cc. of the trichloroacetic acid filtrate were pipetted into a 15 cc. graduated centrifuge tube.   To this was added 1 cc. of Molybdate Solution 2 and 0.4 cc. of the aminonaphtholsulfonic acid reagent.   The contents were diluted to 10 cc. with distilled water, mixed, and allowed to stand 5 to 10 minutes.   The blue color was then compared with a standard.

Acid-Soluble Phosphorus—5 cc. of the trichloroacetic acid filtrate were pipetted into a Pyrex test-tube (25 mm. by 200 mm.); to this were added 2.5 cc. of 10 N H_2SO_4 and a glass bead.   The tube was then heated on a sand bath.   After the water had been driven off, as shown by charring of the contents, 2 or 3 drops of concentrated HNO_3 were carefully added by allowing it to drop down the side of the tube which was held at an incline.   The contents of the tube were then taken down to fumes of SO_3 and if the solution thus remaining was not practically colorless the nitric acid treatment was repeated.   The tube was allowed to

cool somewhat and the walls were washed with a small amount of water, after which the contents were again taken down to fumes of SO_3. (The purpose of washing the walls with a small amount of water was to safeguard against any fumes from the nitric acid which may have condensed on the cooler portions of the tube.) The tube was then allowed to cool and the contents washed into a 25 cc. volumetric flask. To this solution were added 5 cc. of Molybdate Solution 3, and 2 cc. of the reducing reagent. The solution was then made up to volume with distilled water, allowed to stand about 5 minutes, and the color of the solution compared with the standard.

Lecithin Phosphorus (Lipid Phosphorus)—The alcohol-ether solution was made by mixing 75 cc. of alcohol and 25 cc. of ether. 50 or 60 cc. of the solution were placed in a 100 cc. volumetric flask, and 5 cc. of blood were added. The flask was then shaken thoroughly and placed in a beaker of boiling water until the solution boiled. The flask was allowed to cool, the solution was made up to volume with the alcohol-ether mixture, and filtered. 20 cc. of this filtrate were placed in a Pyrex test-tube and evaporated to dryness in a boiling water bath. To the residue were added 5 cc. of 5 N sulfuric acid and the tube was treated exactly as in the procedure for acid-soluble phosphorus.

Ester Phosphorus (Acid-Soluble Organic Phosphorus)—This figure was obtained by subtracting the inorganic phosphate value from the figure for the acid-soluble fraction.

Total Phosphorus—1 cc. of N magnesium nitrate was placed in a small platinum evaporating dish, and to this was added 1 cc. of whole blood. The dish was rotated so as to effect thorough mixing of the nitrate and the blood, and the contents then evaporated to dryness on a sand bath. The dish was placed in the electric furnace and heated until a white ash resulted. The ash was taken up in 5 cc. of 10 N H_2SO_4, and the solution washed through a filter paper into a 100 cc. volumetric flask. To this were added 10 cc. of Molybdate Solution 3 followed by 4 cc. of reducing reagent, and the solution was made up to volume with distilled water. The solution was thoroughly mixed, allowed to stand 5 minutes for color development, and then compared with the standard.

In Table I are shown the results obtained for one calf from each of the six lots. The values for each calf are typical of those ob-

tained for the other animals in the same group. An analysis of these figures shows that the values obtained for different animals on the normal rations and on the rachitogenic rations are exceedingly uniform. In Lots 2 to 5 (normal calves) the inorganic phosphate varies from 6.1 to 6.3 mg. of P and the total phosphorus from 18.8 to 21.1 mg. of P per 100 cc. of blood. The variations in the other fractions are of the same magnitude. These differences are very small when one considers that the calves in the different lots

TABLE I

Phosphorus Partition in Blood of Individual Calves

All values are given as mg. of P per 100 cc. of whole blood.

	Calf No.	Lot No.	Inorganic P	Acid-soluble P	Ester P	Lecithin P	Total P
Normal animals	28	2	6.2	10.0	3.8	9.5	19.7
	22	3	6.2	9.5	3.3	10.3	21.1
	6	4	6.3	9.7	3.4	8.8	18.8
	7	5	6 1	9.8	3.7	9.3	19.2
Rachitic animals	11	1	2.8	6.0	3.2	9.7	15.5
	10	6	2.6	5.1	2.5	9.7	15.2

TABLE II

Effect of Rickets on Phosphorus Partition in Blood

All values are given as mg. of P per 100 cc. of whole blood.

	Inorganic P	Acid-soluble P	Ester P	Lecithin P	Total P
Average for 8 rachitic calves	3.0	5.8	2.8	9.0	15.5
" " 14 normal "	6 2	9.6	3.4	9.7	20.0
Difference......................	3.2	3.8	0.6	0.7	4.5

received the vitamin D supplement in different forms. The values for Lots 1 and 6 (rachitic calves) are equally consistent. It is also interesting to note in the case of the rachitic calves that the blood having the highest inorganic phosphate values also contained the higher amounts of total phosphorus. The uniformity of the figures for inorganic phosphorus is very significant in view of the fact that high results for this fraction are so often recorded for rachitic animals when the blood is allowed to clot before analysis.

The average figures for eight rachitic and fourteen normal calves are given in Table II. The values in the second column demonstrate that rickets in calves is accompanied by a definite and pronounced decrease in the inorganic phosphate fraction in the whole blood. The values decrease from a normal of 6.0 mg. to about 3.0 mg. of P per 100 cc. of blood. The difference in the total phosphorus of normal and rachitic blood is slightly greater than can be accounted for by the change in the inorganic form alone. The difference is due to a slight decrease in the ester and lecithin phosphorus. Since the decrease in total phosphorus during rickets is slightly greater than the decrease in the inorganic fraction and because only 1 cc. of blood is necessary for this determination, the total P content may be more valuable in diagnosing rickets than is the inorganic fraction, especially with small experimental animals, where it is difficult to get sufficient blood from one animal. However, the presence of additional phosphorus compounds in the blood of certain animals may complicate this procedure.

It is interesting to correlate the values obtained for the organic acid-soluble phosphorus in this work with those obtained by Ashford (23) during vitamin D hypervitaminosis in rabbits. The inorganic phosphorus fraction decreases during vitamin D avitaminosis and increases during vitamin D hypervitaminosis, but the changes in the organic forms are small in both cases. It seems improbable, therefore, that the decrease in inorganic phosphorus during rickets can be due to a decrease in the supply of organic ester which can be hydrolyzed into phosphoric acid. Kay (24), however, has shown that there is a decrease in the amount of organic acid-soluble phosphorus in blood during acidosis when the inorganic fraction is increased and that the change is limited to that fraction of the phosphate ester which is not hydrolyzed by bone enzyme. There is, of course, a possibility that the ratio between the ester which is not hydrolyzed and the ester which is hydrolyzed by bone enzyme undergoes some change during rickets.

The total phosphorus values obtained by analysis and by addition of the acid-soluble and lecithin fractions are given for ten of the calves in Table III. Our results differ from those obtained by Walker and Huntsinger (21) for human blood. They found the

total phosphorus by addition to be slightly higher than by analysis, while we found the reverse to be true in practically every case. Malan and Green (25) suggest that the slightly larger amount found by analysis is due to the presence of nucleoprotein contained in the precursors of fully mature erythrocytes in the blood. These workers found the nucleoprotein fraction to be highest in young calves, and that it practically disappeared as the animal became older. A small part of the difference observed in our figures may be due to the presence of traces of nucleoprotein, but we suspect that the largest part of the difference can be explained by small

TABLE III

Comparison of Total Phosphorus by Analysis and by Calculation

All values are given as mg. of P per 100 cc. of whole blood.

Calf No.	Total P		Difference
	By addition	By analysis	
23	15.1	15.8	0.7
43	14.0	14.2	0.2
3	18.1	18.8	0.7
22	19.8	21.1	1.3
48	19.2	19.8	0.6
6	18.5	18.8	0.3
7	19.1	19.2	0.1
39	21.3	21.8	0.5
52	17.0	18.9	1.9
26	15.3	15.7	0.4
Average..			0.67

errors in some of the lecithin determinations. A slight amount of fading was noted when the color was developed in a few of the lecithin determinations, and the greatest difference was observed in these samples. The phosphorus by analysis rarely exceeded that obtained from calculation by more than 3 to 4 per cent when this difficulty was eliminated.

Shortly after the blood samples had been taken, two of the rachitic calves were given aerated cod liver oil in addition to the basal ration. Further blood samples were taken from these calves when they had been on the supplemented diet 2, 3, 4, 5, and 12 weeks, respectively. The results of these analyses are plotted in Chart I.
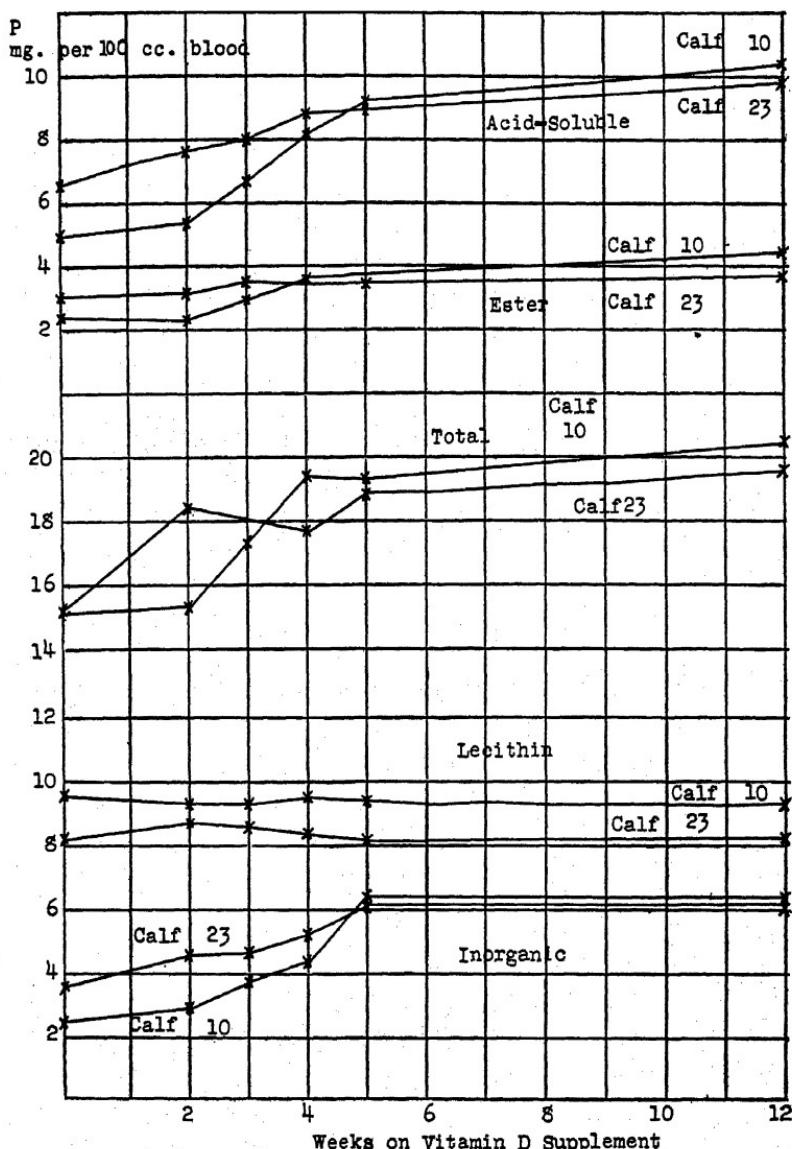
CHART I. The curves in the chart represent the weekly changes in the inorganic, acid-soluble, ester, lecithin, and total phosphorus content of whole blood when aerated cod liver oil was added to the basal ration of rachitic calves. Calf 23 was in Lot 1 and Calf 10 was in Lot 6. All values are given as mg. of P per 100 cc. of whole blood.

These results show that the addition of vitamin D to the ration of rachitic calves causes a gradual increase in all of the phosphorus fractions in the blood except lecithin. The increase is the greatest between the 2nd and 5th weeks after the addition was started. In practically every case the maximum value is reached by the end of the 5th week, when each figure corresponds very well with similar figures for normal calves. The curves for the ester phosphorus verify the figures given for this fraction in Table II. Although the change is slight, the consistently lower figures during rickets and the definite increase when vitamin D is supplied demonstrate that there is a significant change. In Table II the average lecithin value for rachitic calves was slightly lower than that for the normal ones, but the lecithin curves for these two calves fail to show any increase when cod liver oil is supplied.

A study of the distribution of the phosphorus fractions between the plasma and corpuscles was also made on the blood of two rachitic calves, two normal calves, and two rachitic calves that had received vitamin D for 6 weeks. The percentage of corpuscles and plasma in each sample of blood was determined by placing 15 cc. of oxalated whole blood in a stoppered graduated centrifuge tube and centrifuging until the corpuscles were well packed at the bottom of the tube. The volume of the corpuscles and serum was then read and the percentage calculated, their sum being used as the total volume of blood. The plasma and corpuscles were separated very carefully and total phosphorus, acid-soluble phosphorus, and inorganic phosphorus were determined on each of the two portions.

Since the results for the normal calves and the rachitic calves that had received the vitamin D treatment were very similar, the values for only two animals are given in Table IV: Calf 7, which was rachitic, and Calf 10, which had been rachitic but was cured by the addition of cod liver oil to the diet. Values for the total, acid-soluble, and inorganic phosphorus are given for whole blood, 100 cc. of plasma, 100 cc. of corpuscles, and for the plasma and the corpuscles contained in 100 cc. of blood. The latter figures were calculated from the percentage of plasma and corpuscles found in the blood.

The results for whole blood are very similar to those given in Table II, and in most cases agree with those obtained by adding

the amounts found in the plasma and the cells present in 100 cc. of blood. In a few cases, especially the total phosphorus, there is a considerable difference between the two values. This raises a question regarding the actual distribution of the various fractions in the blood as it circulates in the body. The analyses which we have made are based on the separation of the corpuscles and plasma by centrifugation. It is impossible to conclude that the distribution of the phosphorus components between the plasma and corpuscle fractions is the same after they have been separated by centrifuging as before.

TABLE IV

Distribution of Phosphorus between Plasma and Corpuscles

	Whole blood	
	Calf 10 (normal) per cent	Calf 7 (rachitic) per cent
Corpuscles.....................	38	33.7
Plasma.........................	62	66.3

	Inorganic P	Acid-soluble P	Total P	Inorganic P	Acid-soluble P	Total P
	mg. per 100 cc.	*mg. per 100 cc.*	*mg. per 100 cc.*	*mg. per 100 cc.*	*mg. per 100 cc.*	*mg. per 100 cc.*
Corpuscles................	5.20·	12.95	29.40	3.67	10.28	27.20
Plasma....................	7.40	9.43	11.45	3.46	3.76	6.65
Whole blood...............	6.25	9.55	19.35	3.20	6.15	16.36
Calculated values per 100 cc. whole blood						
Corpuscles................	1.98	4.92	11.17	1.24	3.46	9.16
Plasma....................	4.59	5.85	7.10	2.29	2.50	4.42
Whole blood...............	6.57	10.77	18.27	3.53	5.96	13.58

Our results show that the corpuscle fraction obtained by this method contains definite amounts of inorganic phosphorus. It is difficult to decide whether this phosphate is held within the cells or is concentrated in the small amount of plasma adhering to the cells. Buell (26) has shown that there is practically no inorganic phosphate in the corpuscles of normal dog blood. We have been able to reduce the acid-soluble phosphorus of corpuscles from 10 to 11 mg. to about 7 mg. of P per 100 cc. of corpuscles by washing them three times with isotonic salt solution. This decrease is due almost entirely to a loss of inorganic phosphate, and the cells so washed contain only traces of the inorganic fraction. Whether

this phosphate comes from that present in the small film of plasma surrounding the cells or from within the corpuscle, due to a diffusion through the membrane when the concentration of phosphate ions is reduced by the addition of salt solution, we have not determined. In either case the possible error due to this condition would be eliminated by using whole blood rather than plasma. If the phosphorus is held outside the cell, the amount remaining in the plasma after centrifuging depends upon the amount carried down by the cells. If it is held within the corpuscle, the cell membrane must be permeable to the phosphate ion and the decrease during rickets would be equal in the plasma and the corpuscle.

The uniformity of the results which we have obtained when the analyses were carried out on whole blood is undoubtedly due to the fact that the error encountered in the separation of plasma or serum can be eliminated when whole blood is used.

When one is interested in comparing blood phosphorus values from normal and rachitic animals, the simplest and most accurate method is the determination of inorganic phosphorus on whole blood. In the case of calves the total phosphorus may be substituted for inorganic phosphorus, because the relative change in the two fractions is very similar.

SUMMARY

1. Tables are presented (Tables I, II, and IV) to show the phosphorus partition in whole blood of rachitic and non-rachitic calves. Rickets produced experimentally by a vitamin D deficiency causes a decided decrease in inorganic phosphorus, which causes a decrease in the acid-soluble and total phosphorus. The decrease in total phosphorus is slightly greater than can be accounted for by the change in inorganic phosphorus alone, due to a slight but definite decrease in ester phosphorus and possibly a slight decrease in lecithin phosphorus.

2. Exceedingly uniform results are obtained for inorganic phosphorus in rickets when the analyses are made on whole blood and carried out immediately after the blood is drawn.

3. When the diet of rachitic calves is supplemented with vitamin D the decreased phosphorus fractions gradually return to normal values.

4. The total phosphorus by analysis was found to be slightly higher than the total phosphorus by addition of the acid-soluble and lecithin fractions. The difference in the majority of the cases is less than 3 to 4 per cent of the total phosphorus by analysis.

5. The distribution of the various phosphorus fractions between the plasma and the corpuscles of blood from normal and rachitic calves is given. Evidence is presented to show that the determination of inorganic phosphorus in plasma does not give a complete picture of the total change in the inorganic phosphate fraction during rickets.

We wish to express our appreciation to Professor I. W. Rupel of the Animal Husbandry Department for his cooperation in these studies.

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ANIMAL CALORIMETRY

THE RESPIRATORY METABOLISM OF EXERCISE AND RECOVERY IN DEPANCREATIZED DOGS*

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The overproduction theory of diabetes sponsored by von Noorden, in 1905, depends upon the premise that carbohydrate is produced from fat and is the sole fuel for muscular contraction (Macleod (1)). This theory has recently attracted renewed attention, according to editorials appearing in the *Journal of the American Medical Association* (2), although evidence that the respiratory quotient of normal and of diabetic muscle is less than unity has been found by many investigators (Peserico (3), Takane (4), Himwich and Castle (5), Himwich and Rose (6), Richardson, Shorr, and Loebel (7)). A full discussion of the significance of these results is given in the reviews of Richardson (8), of Rapport (9), and of Cori (10).

In support of von Noorden's theory Chaikoff and Macleod (11) have interpreted the increase in R.Q. which they found on exercising depancreatized dogs as indicating the ability of the diabetic animal to oxidize sugar. They caused muscular activity by exposure to low temperature. For the first 30 minutes of shivering the R.Q. rose above the resting diabetic level (0.68 to 0.70) but returned again in most of the cases to approximately the basal value during the 2nd or 3rd hour of hyperactivity. No observations of post-work recovery periods are reported. The authors have considered a single control experiment on the CO_2 capacity of the blood as sufficient evidence for discarding the "blowing off" of CO_2 as an explanation of the rise in R.Q., but have attributed the results to an oxidation of carbohydrate.

Only two other reports have been found concerning the effect of exercise on the respiratory metabolism of depancreatized dogs. The first experiment was conducted by Mohr (12), in which the R.Q. was elevated from 0.708 to 0.837 while the animal was running on a treadmill at a rate that increased the O_2 consumption 9-fold. A reference to an unpublished experiment is cited by Rapport ((9) p. 437). The R.Q. of the combined exercise and recovery was 0.71, the same as that of the previous resting period. No records of separate respiratory quotients after work have been found.

The importance of the recovery period should be emphasized. The ability of depancreatized dogs to pour large amounts of lactic acid into the blood stream has been demonstrated by Weber, Briggs, and Doisy (13). Himwich, Loebel, and Barr (14), also Hetzel and Long (15) have reported increases in blood lactic acid during exercise in diabetes mellitus. The latter authors noted a retention of CO_2 below the resting level in the later stages of recovery but did not attempt to evaluate the observation. Recently the experiments of Kilborn (16), of Ferguson, Irving, and Plewes (17), and of Shaw and Messer (18) have called attention to the importance of the tissues as well as the blood in the "blowing off" of CO_2 from the body. The blood accounted for only about 10 per cent and the muscles about 40 per cent of the total CO_2 removed by overventilation, according to Irving, Ferguson, and Plewes (19). Additional evidence in this regard has been furnished by Martin, Field, and Hall (20), who have demonstrated a greater concentration and a longer retention of lactates in the muscle than in the blood after exercise. In a study of blood changes in diabetic dogs (Himwich, Chambers, Koskoff, and Nahum (21)) some unpublished observations were made, which showed a rise in R.Q. during exercise, accompanied by an increase in blood lactates, but with no appreciable change in the blood alkali reserve.

It seemed to us that further studies of the recovery process in depancreatized dogs should be made before an increase in R.Q. could be definitely associated with the oxidation of carbohydrate.

The data which form the subject matter of this paper were obtained in two ways. In one series of experiments in the respiration calorimeter unanesthetized animals were stimulated to muscular activity by the injection of large doses of epinephrine, as suggested by the work of Lusk (22). In this procedure the exercise period can only be observed but not controlled.

Therefore, another series was conducted to separate the respiratory effects occurring during the exercise period from those dependent upon recovery. For this reason the muscular activity was regulated by electrical stimulation and the animals were, therefore, anesthetized. Additional information concerning recovery in the diabetic animal was obtained from following the blood lactic

acid and glucose changes coincident with the respiratory metabolism, hence the Kitchen mask (23) and Benedict Universal apparatus were used instead of the respiration calorimeter. Since no studies of recovery in normal fasting animals could be found for comparison with the fasting diabetics, similar experiments were performed on the same dogs before the pancreatectomy. A number of the dogs were exercised on the treadmill as a control on the effect of the amytal anesthesia on blood lactic acid and glucose changes. The latter data are included in another publication (24).

Methods

Adult mongrel female dogs weighing between 10 and 20 kilos were used for these experiments. The pancreas was removed in one operation under amytal anesthesia. The animals were fasted for 24 to 48 hours before operation and received no food afterward with the two exceptions noted in Tables VIII and IX. By observing strict aseptic technique good postoperative recovery was obtained without insulin or food, such that in several instances the dogs ran on the treadmill on the 3rd day after the removal of the pancreas.

The electrical stimulation experiments under amytal anesthesia were performed on the 3rd to the 5th postoperative day, essentially according to the procedure outlined, in Protocol 1, for a typical experiment.

Amytal narcosis was used for the reasons discussed in an earlier paper (25), the dose being 50 to 60 mg. per kilo of body weight, injected intraperitoneally. As indicated in Protocol 1, the respiratory gases were measured for the most part in 20 minute periods, succeeded by 10 minute intervals in which the mask was open to room air in order to change the absorbing bottles. The figures in Tables II, III, IV, and X, therefore, represent the metabolism in 30 minute periods, as calculated from 20 minute determinations. The respiratory gases were collected for several preliminary periods until a basal level was established. The R.Q. was usually low during the 1st hour after the amytal, a point which was missed in the earlier calorimeter experiments (26), as this time was used for preliminary adjustment. Thereafter the R.Q. remained stationary, with only minor fluctuations. The total metabolism also was depressed from 10 to 15 per cent for the 1st hour; therefore, the results for this period have not been included.

A strong contraction of all the leg muscles was produced twice per second for 1 hour by passing an induced tetanizing current through the electrodes, one pair of which were attached to the fore feet and another pair to the hind feet. The diabetic dogs showed an increased heat production for the work and recovery periods of about 33 calories, which was equivalent to a treadmill run of approximately 1 mile.

Simultaneously with the shutting off of the stimulating current the first post-work respiration period was started, and blood was

PROTOCOL 1

Procedure for a Typical Electrode Stimulation Experiment (Dog 79)

	Respiratory gases measured	Blood drawn	Urine by catheter
		9.20 a.m.	
Amytal, 9.30 a.m.			
			9.54 a.m.
	10.40 a.m.–11.10 a.m.		
	11.20 " –11.40 "		
	11.45 " –12.05 p.m.	11.55 a.m.	
	12.25 p.m.–12.45 "		12.53 p.m.
Work started, 1.18 p.m.	1.18 " – 1.38 "	1.40 p.m.	
	1.47 " – 2.07 "		
Work stopped, 2.18 p.m.	2.18 " – 2.38 "	2.20 "	2.36 "
	2.48 " – 3.08 "		
	3.18 " – 3.38 "	3.15 "	
	3.51 " – 4.11 "	4.15 "	
	4.39 " – 4.59 "		4.50 "
	5.15 " – 5.45 "		
	5.55 " – 6.15 "		6.02 "

then drawn as quickly as possible from a leg vein. The time of taking the other blood samples and of collecting the urine is shown in Protocol 1. Post-work observations were carried on until the oxygen consumption returned to approximately a constant value, which was usually about 10 per cent higher than the basal metabolism. External heat was applied to the body when necessary to maintain the normal body temperature. The increased heat production under these conditions agrees with the results of Hunt and Bright (27) in cats under amytal anesthesia.

For the normal control experiments the dogs were fasted for 3

to 6 days to bring them to a nutritive condition comparable with that after pancreatectomy and fasting. The same procedure described above for the stimulation experiment was then followed.

The technique of the other series of experiments differed from the first in that muscular activity was induced by the injection of epinephrine, the animals were not anesthetized, and the respiratory gases were collected continuously. After the determination of the basal metabolism in the respiration calorimeter the epinephrine (Armour and Company) was injected subcutaneously and intramuscularly and the animal was returned to the calorimeter for observation in hourly periods. Owing to the necessary preliminary adjustments 30 to 45 minutes of activity ensued after the injection before the 1st hour of observation was started. Usually the animal was very active for the first 2 hours and then gradually became quiet during the later periods. In the earlier experiments the large dose of epinephrine, 0.5 mg. per kilo, proved to be fatal for the depancreatized dogs. Later 0.25 mg. per kilo was used and in some cases caused as great an increase in heat production as the larger amount.

The condition of the animals with regard to sugar elimination prior to the experiments, given in Tables II to IX, is shown by the 24 hour urinary D:N ratios in Table I. No attempt was made to start the collection of urine with the first appearance of the glycosuria; therefore, the ratios for the 1st postoperative day are not significant.

The chemical methods employed were the Shaffer-Hartmann for blood glucose, the Friedemann-Kendall for lactates, the Benedict for urinary sugar, and the Kjeldahl for urinary nitrogen.

Necropsy was performed on all dogs. We are indebted to Dr. J. F. Nonidez, of the Department of Anatomy, for the microscopic examination of suspicious tissue.

<center>EXPERIMENTAL</center>

<center>*Electrode Stimulation Series*</center>

Tables II, III, and IV contain the data from experiments on three anesthetized dogs in which the muscular work was caused by stimulation with an electric current, thus definitely separating the exercise and recovery periods. In Dog 79 (Table II), following an

TABLE I

Daily Urinary D:N Ratios for 24 Hour Periods after Pancreatectomy

Period No.	Dog 79	Dog 2	Dog 7	Dog 63	Dog 73	Dog 66	Dog 78	Dog 70
1				0.68				3.08
2	2.36	1.75	3.25	3.29	2.57		3.06	2.77
3	2.77	2.92	3.22	3.03	2.61	2.89	3.06	2.92
4	2.73		4.02	3.30*	2.16		2.98	3.12
5	2.65			2.93*	3.07		3.15	2.90
6					3.40		3.26	2.34
7					2.79		3.69	2.34
8							3.28	1.94
9							3.41	
10							3.41	
11							3.13	
12							6.07†	
13							3.10	

* Exercise on treadmill.

† After glucose.

TABLE II

Metabolism of Dog 79 on 5th Day after Pancreatectomy

Weight 14.1 kilos.

30 min. periods	O₂	Urine		R.Q.	Indirect heat	Blood	
		N per hr.	D:N			Glucose	Lactic acid
	gm.	*mg.*			*calories*	*mg. per cent*	*mg. per cent*
Pre-work	4.54			0.67	14.60	245*	9*
	4.70			0.72	15.12	245	7
	4.75			0.74	15.29		
Average...	4.66	263	2.94	0.71	15.04		
Work	9.16			0.92	29.67	263	11
	10.35	371	3.49	0.81	33.57	201	14
Post-work	5.66			0.66	18.28		
	5.49			0.64	17.73	212	8
	5.50			0.65	17.76		
	5.28			0.72	17.04	223	11
	5.17	191	2.66	0.76	16.68		
	4.88			0.70	15.72		
	5.16	434	2.46	0.73	16.64		

* Before amytal anesthesia.

average basal quotient of 0.71, the R.Q. rose during the exercising period to an average of 0.86 and the blood lactic acid to 14 mg. per 100 cc.   For the first three half hours after work the R.Q. was below the basal level, remaining at about 0.65, then returned to 0.73 for the remainder of the observed time.   The heat production was approximately doubled during stimulation and did not return to a constant level for from 2 to 3 hours after the work ceased.

TABLE III

Metabolism of Dog 2 on 3rd Day after Pancreatectomy

Weight 11.1 kilos.

30 min. periods	O₂	Urine		R.Q.	Indirect heat	Blood	
		N per hr.	D:N			Glucose	Lactic acid
	gm.	*mg.*			*calories*	*mg. per cent*	*mg. per cent*
Pre-work	3.10			0.68	9.95	255	21
	3.40			0.72	10.94		
	3.85			0.68	12.41		
	3.96			0.68	12.78		
	4.09			0.74	13.20	298	17
Average...		199	2.92	0.70			
Work	6.83			0.79	22.15	287	45
	8.57	267	3.77	0.74	27.86	310	26
Post-work	4.18			0.80	13.41		
	4.18			0.62	13.41		
	3.90			0.71	12.49	272	21
	3.70			0.69	11.83		
	3.67			0.72	11.73		
	3.81	306	4.50	0.72	12.19	280	24

The resting blood glucose concentration was not significantly changed by the anesthetic but fluctuated during the stimulation when a greater excretion of sugar occurred.   The dog recovered completely and performed a treadmill experiment 2 days later.

In the experiment on Dog 2 (Table III) the lactic acid reached a high level during work, 45 mg. per cent, but the recovery process brought it back to the pre-work range and the animal survived. For the first 20 minutes after exercise the R.Q. was high, in this

regard resembling the results obtained by Carpenter and Fox in the 30 minute work experiments on normal man (28).   The following period the low R.Q. was encountered.   The heat production of the pre-work periods was probably abnormal, as it rose rapidly and surpassed the basal level indicated by the recovery figures.

The smaller differences in quotients found in Dog 7 (Table IV) are of interest because a lack of recovery is indicated by the continuously high lactic acid concentration in the blood.   The blood glucose also was high and continued to rise until the animal died

TABLE IV

Metabolism of Dog 7 on 4th Day after Pancreatectomy

Weight 15.8 kilos.

30 min. periods	O_2	R.Q.	Indirect heat	Blood	
				Glucose	Lactic acid
	gm.		*calories*	*mg. per cent*	*mg. per cent*
Pre-work	5.84	0.73	18.54		
	6.03	0.75	19.17		
	6.00	0.75	19.07	430	32
Average.....	5.96	0.74	18.93		
Work	9.64	0.79	31.01	472	47
	10.54	0.75	33.96	518	42
Post-work	6.70	0.67	21.37		
	6.34	0.69	20.18	528	46
	6.20	0.73	19.72		
	6.17	0.71	19.63		
	5.02	0.68	15.85	572	50

at the end of the fifth post-work period.   The latter fact probably explains the decreased oxygen consumption and lowered R.Q. of this period.

Epinephrine Series

A typical epinephrine reaction is illustrated by the experiment of March 28 on Dog 63, shown in Table V.   During the 1st hour of activity the R.Q. rose to 0.76, then fell to an average of 0.72 for the next 2½ hours.   There was no movement in the last period during which the R.Q. dropped to 0.67, thus compensating for the earlier in-

crease.   The average quotient (0.72) for the whole period of muscular movement and subsequent recovery is approximately the same as that of the basal experiment on March 27.   The first adrenalin experiment (March 27, Table V) merely shows the high quotient (0.79) obtained during a shorter period of continuous activity,

TABLE V

Stimulation by Epinephrine in Large Doses

Dog 63, weight 17.7 kilos, depancreatized March 22.

Period ending	O₂	Urine		R.Q.	Calories per hr.		Activity
		N per hr.	D:N		Indirect	Direct	
Basal							
	gm. per hr.	*gm.*					
Mar. 27 1.40 p.m.*.....	11.61	0.467	3.25	0.71	37.13	36.67	Slight movement
Epinephrine, 0.5 mg. per kilo							
Mar. 27 5.33 p.m.†.....	17.81	0.493	8.23	0.79	58.72	60.97‡	Very active
Epinephrine, 0.6 mg. per kilo							
Mar. 28 11.56 a.m.......	13.90	0.445	3.21	0.76	45.11	41.07‡	Some movement
2.32 p.m.†.....	13.78			0.72	44.28	42.43	Active
3.32 "	14.90	0.534	2.80	0.67	47.77	44.31	Quiet
Average.......................				0.72	45.12	42.53	

* Average of 2 hours.

† 2½ hour period.

‡ No body temperature correction.

the amount of which can be judged by the increase in heat production from 37 to 59 calories.   On March 25 and 26 the animal ran on the treadmill for about 3 miles each day.   The high urinary D:N ratio of 8.23 after the first dose of epinephrine indicates that the running had not exhausted the glycogen.   After the second injection no extra sugar was excreted (D:N = 2.80) and the animal was unable to recover.

Another experiment with the larger dose of the hormone was performed on Dog 73 (Table VI) 8 days after extirpation of the pan-

TABLE VI

Stimulation by Epinephrine, Voluntary Activity

Dog 73, weight 12.2 kilos, depancreatized April 10.

Period ending	O₂	Urine		R.Q.	Calories per hr.		Activity
		N per hr.	D:N		Indirect	Direct*	
Basal							
	gm. per hr.	*gm.*					
Apr. 17							
12.00 n..........	6.62	0.325	2.69	0.75	21.44	21.01	Quiet
1.00 p.m........	8.37			0.72	27.08	22.57	Little movement
2.00 "	7.11	0.257	2.36	0.71	22.82	21.69	Quiet
Average.........................				0.73	23.78	21.76	
Epinephrine, 0.5 mg. per kilo							
Apr. 17							
4.20 p.m........	15.94			0.77	52.51	38.20	Very active
5.20 "	15.43			0.69	49.95	41.46	" "
6.20 "	13.13			0.74	42.83	41.75	Active
7.20 "	10.95	0.321	5.68	0.71	35.25	35.50	Some movement
Average.........................				0.73	42.23	37.30	
"Basal"							
Apr. 14							
12.19 p.m........	10.43			0.69	33.60	30.97	Active
1.49 "	10.69			0.74	34.71	31.67	"
2.49 "	7.63			0.78	24.89	26.80	Quiet
3.49 "	9.32	0.306	3.50	0.71	29.98	31.53	Urinated
Average.........................				0.73	30.77	30.24	

* No body temperature correction.

creas.   Although there was some movement throughout the entire period of observation, the average R.Q. (0.73) after the injection was the same as that of the basal metabolism determined earlier

on the same day. An interesting observation of voluntary exercise is included in Table VI. On April 14 a basal metabolism experiment was attempted, but the dog was restless and moved sufficiently during the 1st, 2nd, and 4th hours to increase the heat output about 50 per cent. Although the quotients for the individual hours fluctuate, the average for the whole period (0.73) is the same as that of the basal and adrenalin experiments.

TABLE VII

Stimulation by Epinephrine

Dog 66, weight 15.5 kilos, depancreatized April 20.

Period ending	O₂	Urine		R.Q.	Calories per hr.		Activity
		N per hr.	D:N		Indirect	Direct	
Basal							
	gm. per hr.	*gm.*					
Apr. 23 3.40 p.m.*.....	10.89	0.348	4.78	0.69	35.03	37.86	Slight movement
Epinephrine, 0.25 mg. per kilo							
Apr. 24							
12.00 n.........	19.15			0.75	62.48	66.97†	Very active
1.15 p.m.......	15.45			0.74	50.16	60.00	" "
2.15 "	13.64			0.68	43.70	49.48	Some movement
3.15 "	12.22			0.60	39.05	43.27	Quiet
4.15 "	13.12	0.505	9.82	0.72	42.17	45.38	"
Average.......................				0.70	47.51	53.02	

* Average of 2½ hour period.
† No body temperature correction.

Better results were obtained in Dog 66 (Table VII) with a smaller amount of the stimulant. The high R.Q. (0.75 and 0.74) of the first 2 hours of activity was followed by a compensating fall to 0.60 during the 4th hour of quiet recovery and a subsequent return to 0.72. The average of the 5 hours after the epinephrine (0.70) was approximately the same as the resting quotient of 0.69 on the previous day.

As a control experiment on the ability to oxidize carbohydrate, 16 gm. of glucose (approximately 1.7 gm. per kilo) were given to Dog 78 on the 12th day of fast after the removal of the pancreas. The average R.Q. of 0.72 (Table VIII) for the 2nd, 3rd, and 4th

TABLE VIII

Stimulation by Epinephrine, Metabolism of Ingested Glucose

Dog 78, weight 13.0 kilos, depancreatized June 6.

Period ending	O$_2$	Urine		R.Q.	Calories per hr.		Activity
		N per hr.	D:N		Indirect	Direct	
	gm. per hr.	*gm.*					
June 16							
2.45 p.m.......	6.68			0.71	21.33	22.68	Quiet
3.45 "	6.58			0.76	21.32	22.49	"
4.45 "	6.66	0.288	3.46	0.71	21.31	22.01	"
Average........................				0.73	21.32	22.39	
Glucose, 16 gm.							
June 17							
10.16 a.m.*.....		0.243	2.98				
12.16 p.m.......	7.20			0.70	22.99	23.87	Slight movement
1.16 "	6.87			0.72	21.99	22.07	Quiet
2.16 "	6.69	0.300	12.70	0.74	21.49	22.50	"
Average........................				0.72	22.16	22.81	
Epinephrine, 0.25 mg. per kilo							
June 19							
11.53 a.m.......	9.71	0.233	3.10	0.79	31.95	32.78†	Movement
12.53 p.m.......	9.72			0.66	31.26	29.27	"
1.53 "	7.88			0.73	25.40	25.59	Little movement
2.53 "	7.33			0.73	23.59	23.93	Quiet
3.53 "	7.56	0.299	7.92	0.75	24.50	22.91	Little movement
Average........................				0.73	27.26	26.90	

* 16 gm. of glucose were ingested at 10.16 a.m.

† No body temperature correction.

TABLE IX

Stimulation by Epinephrine

Dog 70, weight 13.3 kilos, depancreatized December 2.

Period ending	O₂	Urine		R.Q.	Calories per hr.		Activity
		N per hr.	D:N		Indirect	Direct*	
	gm. per hr.	*gm.*					

Basal

Dec. 9							
4.20 p.m........	8.81			0.69	28.02	27.03	Quiet
5.20 "	8.54	0.378	2.56	0.74	27.51	25.84	Little movement
Average.........				0.71	27.76	26.44	
Dec. 10							
11.48 a.m........	7.93			0.75	25.79	21.50	Slight movement
12.48 p.m........	8.51	0.276	1.45	0.72	27.43	23.25	" "
Average.........				0.73	26.61	22.38	

Epinephrine, 0.25 mg. per kilo

Dec. 10							
3.04 p.m........	11.95			0.77	39.18	42.37	Very active
4.04 "	10.47			0.79	34.46	37.25	Active
5.04 "	10.34			0.70	33.26	33.48	Some movement
6.04 "	9.82			0.71	31.56	30.15	Quiet
7.04 "	9.73	0.322	8.26	0.71	31.28	28.26	"
Average.........				0.74	33.95	34.30	

Dec. 10, 7.30 p. m., 200 gm. meat

Basal

Dec. 11							
3.17 p.m........	8.56		2.77	0.74	27.62	21.68	Quiet
Dec. 12							
12.35 p.m........	7.88		1.12	0.75	25.58	20.25	Quiet
1.35 "	8.20			0.75	26.63	23.80	"
2.35 "	8.74			0.75	28.42	29.22	Movement
Average.........				0.75			

' No body temperature correction.

hours after the ingestion of the sugar is practically the same as that of the basal metabolism (0.73) on the preceding day. Before the administration of the glucose the D:N ratio was 3.13, and 0.724 gm. of glucose was excreted per hour. 24 hours later the D:N ratio had returned to 3.10 and the urinary sugar excretion was identical (0.724 gm.) with that of the fore period. The recovery of extra glucose was 103 per cent if calculated on the basis of an average D:N ratio of 3.12, or 98 per cent if computed from the extra sugar above the basal value of 0.724 gm. per hour. The average R.Q. during exercise 2 days later (June 19, Table VIII) was not appreciably higher than that of the two previous experiments.

The results given in Table IX illustrate those found in two dogs in which the basal R.Q. has been higher than the theoretical diabetic level, although a comparison of the basal and exercise quotients does not indicate an elevation during work and recovery. In Dog 70 between December 9 and 12 (Table IX) the basal quotient gradually rose from 0.71 to 0.75 and the D:N ratio fell to 1.12. On the latter day the blood contained 416 mg. per cent of sugar. The animal exhibited signs of abdominal distress and increasingly frequent vomiting from the 4th postoperative day until death under amytal anesthesia on December 14. Postmortem examination revealed an extensive colonic intussusception. No evidence of pancreatic tissue was found grossly or in the microscopic examination of three suspicious nodules of tissue. Immediately after death, samples of liver, and striated and cardiac muscle were examined for glycogen, sugar, and lactic acid and found to be essentially the same in carbohydrate content as those of the other diabetic dogs which were studied (Table IX).

In view of these results we would consider the falling D:N ratio in such experimental animals as indicating a retention and not a combustion of sugar, and the rising R.Q. as a reflection of the attendant pathological conditions rather than an incomplete diabetes.

Normal, Fasting Dogs

Two typical experiments on fasting dogs under otherwise normal conditions are shown in Table X. The rise in R.Q. during exercise and the decrease below the resting level during the early recovery

periods are characteristic of all the normal animals except one. In the experiment on Dog 2 (Table X) the post-work fall in R.Q. was delayed until the second 30 minute period, as occurred in this dog when diabetic (Table III). The average figures from eight experiments for blood lactates and glucose in mg. per cent are:

	Rest	Work		Recovery	
		½ hr.	1 hr.	1 hr.	2 hrs.
Lactic acid......................	8	14	13	9	9
Glucose.........................	70	70	68	67	67

TABLE X

Metabolism of Normal Fasting Dogs

30 min. periods	Dog 2, weight 12.9 kilos, 3 day fast					Dog 8, weight 9.2 kilos, 6 day fast				
	O₂	R.Q.	Indi-rect heat	Blood		O₂	R.Q.	Indi-rect heat	Blood	
				Glu-cose	Lac-tic acid				Glu-cose	Lac-tic acid
	gm.		*calories*	*mg. per cent*	*mg. per cent*	*gm.*		*calories*	*mg. per cent*	*mg. per cent*
Pre-work	2.46	0.72	8.27			2.23	0.77	7.49	84	5
	3.14*	0.73	10.55*	43	11	2.32	0.70	7.80		
	2.47	0.72	8.30							
Average...	2.47	0.72	8.28			2.28	0.74	7.65		
Work	8.05	0.78	27.05	45	16	7.24	0.87	24.33	89	8
	5.46	0.86	18.35	48	12	5.44	0.87	18.28	86	12
Post-work	3.60	0.71	12.10			2.95	0.65	9.91		
	3.48	0.66	11.69	38	10	2.85	0.64	9.58	89	8
	3.41	0.69	11.46			2.78	0.70	9.34		
	3.33	0.73	11.19			2.63	0.74	8.84	89	
	3.35	0.70	11.26	43	7	2.65	0.76	8.90		

* Not included in the average since a basal metabolism of 8.28 calories was confirmed in another experiment.

Tissue Analyses

Three of the animals (Dogs 7, 8, and 9) died during the course of the respiration experiments at the times noted in Table XI. Tissue samples from the leg muscles, liver, heart, and diaphragm

were removed for the determination of glycogen according to the technique previously described (25). Soluble carbohydrates and lactic acid were also determined and indicate the extent of post-

TABLE XI

Carbohydrate Content of Tissues of Depancreatized Dogs Expressed in Mg. Per Cent

	Before work			After work	
Dog No.	26	70	9	8	7
Days after pancreatectomy	7	12	4	5	4
Blood					
Glucose	343	607	305	172	572
Lactic acid	21	64	46	51	50
Muscle					
Glycogen	40	109	63	72	152
Soluble carbohydrate	141	179	125	88	117
Lactic acid	31	49	61	63	105
Total	212	337	249	223	373
Liver					
Glycogen	96	87	54	62	46
Soluble carbohydrate	189	493	335	301	636
Lactic acid	19	50	38	52	62
Total	304	630	427	415	744
Heart					
Glycogen		1739	1480	1075	736
Soluble carbohydrate		245	355	177	343
Lactic acid		129	134	159	201
Total		2118	1969	1411	1279
Diaphragm					
Glycogen			43	183	
Soluble carbohydrate			116	87	
Lactic acid			49	90	
Total			208	360	

Dog 26, tissue excised 1½ hours after amytal anesthesia. Dog 70 died 8 minutes after amytal anesthesia. Tissues and blood were taken 2 to 15 minutes post mortem. Dog 9 died 3½ hours after amytal anesthesia. Tissues were taken 24 to 33 minutes post mortem. Dog 8 died 10 minutes after work. Tissues were taken 13 to 24 minutes post mortem. Dog 7 died 3 hours after work. Tissues were taken 19 to 31 minutes post mortem.

mortem glycolysis. The tissue carbohydrate data on these three dogs together with similar results on two others are presented, in Table XI, with the blood glucose and lactate figures for compari-

son. As noted above, the blood lactate in Dog 7 (Table IV) remained at about 50 mg. per cent after the exercise. A similar high value was found in Dogs 8 and 9 before death, as compared to the more normal figure of 21 mg. per cent in Dog 26. "Total" carbohydrates in the muscle and liver were consistently lower than in fasted dogs (25) but were sufficient in amount to indicate that death was not due to exhaustion of glucose or glycogen. In the two dogs in which the diaphragm was analyzed, the total carbohydrate values of 208 and 360 mg. per cent resemble the striated muscle and give no evidence of the ability to store glycogen which the cardiac muscle possesses. In this regard the diabetic dog is similar to the normal one (25).

Alcohol Checks

The same degree of accuracy of the calorimeter reported in the other papers of this series has been found in the ten alcohol checks controlling this part of the work. The R.Q. for each experiment of 3 or 4 hours duration (alcohol checks, Nos. 272 to 275, 282 to 284, 293 to 295) was as follows: 0.665, 0.659, 0.652, 0.669, 0.667, 0.677, 0.667, 0.660, 0.657, and 0.664. The average R.Q. for the ten checks was 0.664, and the average calculated heat was 24.52 calories per hour as compared to the direct measurement of 24.19 calories per hour.

DISCUSSION

The results from the two methods of stimulating muscular exercise supplement each other in demonstrating that an elevation of the R.Q. occurred during exercise and a compensatory fall afterwards such that the combined periods of activity and recovery showed no increase over the basal level.

The rise in R.Q. while the muscles of the anesthetized dogs were stimulated, and for the 1st hour of observation after the epinephrine injection, was of about the same order as that found by Chaikoff and Macleod (11) during the first half hour of shivering.

After exercise, the fall in R.Q. below the resting level was apparent in all three of the electrode stimulation experiments, although it was most marked in the case of Dog 79 (Table II), which was in the best physical condition, if judged by the resting lactic acid concentration of the blood. It is hardly valid in this series to calcu-

late a balance between the increased R.Q. during work and the subsequent decrease, since the respiratory gases were not collected continuously, but for 20 minutes of each 30 minute period. However, in the other series the average R.Q.'s after epinephrine (Tables V to IX) show the total respiratory exchange of the animals for the 5 hour period during which the respiration box was sealed. Technical errors due to changing conditions are therefore minimized. It is significant that in each of the five experiments given in Table XII the average R.Q. of the post-epinephrine period is practically the same as the average for the basal periods.

To what extent the results of the muscular activity in the latter series were complicated by the effect of the epinephrine *per se* can only be surmised. The elevation of the quotient after epinephrine

TABLE XII

Average Respiratory Quotients

Dog No.	Basal	Epinephrine
63	0.71	0.72
73	0.73	0.73
66	0.69	0.70
78	0.73	0.73
70	0.73	0.74
Average............	0.72	0.72

in clinical diabetes was attributed by Bornstein and Müller (29) to the elimination of extra CO_2 through an increased pulmonary ventilation. Similar effects were obtained by Lyman, Nicholls, and McCann (30) and by Boothby and Sandiford (31). However, in two depancreatized dogs Soskin (32) found no significant change in R.Q. after a small amount (0.1 mg. per kilo) of the hormone. While this result was confirmed by Chaikoff and Weber (33) in two experiments, another animal showed a definite rise from 0.683 to 0.747. In a later experiment the R.Q. increased from 0.704 to 0.857 during the 1st hour after the injection when the dog was much excited and moving about. Subsequent doses had little effect on the quotient when the subject was quiet and caused, if anything, a slight depression, as the average was 0.682 for the entire period, exclusive of the 1st hour "during which the animal was undoubtedly blowing off CO_2." Including the 1st hour, the average was 0.707, which is practically identical with that of the

3 hour resting control period (0.704) on the preceding day. Cori (10) has recently reviewed the literature on the production of lactic acid from muscle glycogen by adrenalin in the normal animal. An indication for a similar action in diabetes is found in the work of Geiger and Schmidt (34) on phlorhizinized dogs. From these considerations it seems probable that the reaction of increased respiration and lactic acid formation is much the same whether from epinephrine or from muscular exercise and that the former would to this extent augment the latter.

These respiratory data are in accord with the observations of Grafe and Salomon (35) and of Richardson and Levine (36) that light exercise did not change the R.Q. of incompletely diabetic patients. They are also correlated with the results of Rapport and Ralli (37) on phlorhizinized dogs. In the work of both Richardson and Levine and of Rapport and Ralli sufficient time was allowed after cessation of muscular effort for stabilization of the respiration box. Thus the exercising R.Q. represents an average of a working period and a recovery period of approximately equal duration.

Several interesting points concerning the recovery phase in the diabetic animal are brought out in the comparison with the normal fasting dogs. The increased blood lactic acid resulting from muscular activity in the diabetic was anticipated from the work of Weber, Briggs, and Doisy (13), of Himwich, Loebel, and Barr (14), and of Hetzel and Long (15). That the formation and resynthesis may be quite similar to the normal is seen in comparing the figures for Dog 79 (Table II) with the averages for the normals, also with Table X. In Dog 2 (Table III), in spite of a greater increase in blood lactate in the first 30 minutes of exercise, the return to the resting level was not abnormally retarded. This point is more fully established by the results obtained on the same dogs when not anesthetized. The details are published separately (24); the average figures for blood lactates in mg. per cent are as follows:

	Rest	Work		Recovery	
		½ hr.	1 hr.	1 hr.	2 hrs.
Normal fasting...................	12	20	15	6	6
Depancreatized	12		16	11	10

While the differences are small, some indication of a slight delay in reconversion is seen in these average recovery values of 6 and 6 mg. per cent of blood lactates in the normal fasting state and 11 and 10 in the depancreatized condition.

Although the effect of exercise on blood lactates was quite similar in the normal and the diabetic condition, a marked difference was apparent in the blood glucose changes. In the normals there was practically no variation in blood glucose throughout the experiment, whereas in the diabetic animals a fluctuation of 40 or 50 mg. per cent occurred. The relation of these changes to the amount of glucose excreted is discussed in another article (24).

Additional evidence of a slower recovery process in the diabetics than in fasting normals is obtained from a calculation of the excess metabolism after stimulation. Inasmuch as there was a variation in the amount of work done in the different experiments, the total excess heat produced during the post-work periods is expressed as a percentage of the total work, computed in calories from the extra oxygen consumption over the basal level during the work and post-work periods. In the two depancreatized dogs in which recovery was complete, after stimulation ceased the recovery heat was 11.0 per cent for Dog 79 and 13.7 per cent for Dog 2, whereas in the fasting control experiment on the latter (Table X) only 5.5 per cent was delayed. Four other control experiments were similar, yielding, respectively, 7.6, 4.3, 7.3, and 5.2 per cent, or an average of 6.0 per cent. Dog 5 was the exception, giving the unaccountably high value of 20.9 per cent. These figures on recovery heat in the intact diabetic animal are of interest in connection with the recent work on glycogen resynthesis and recovery in diabetic skeletal muscle, reviewed by Cori (10) and by Milroy (38).

It seems unlikely, according to the blood lactates, that the amytal seriously interfered with the recovery process. Martin, Field, and Hall (39) have recently reported no significant differences in recovery between amytalized dogs and those with the brain stem transected.

It is our interpretation that the evidence presented in this paper is strongly against the oxidation of carbohydrate by the exercising depancreatized animal. The electrode stimulation experiments, in which the work and post-work effects are separated,

show that an increased concentration of lactic acid in the blood is associated with a rise in quotient during the period of muscular activity and that after work the blood lactate returns to the basal level while the R.Q. falls below and then regains the resting value. In the epinephrine series the R.Q. of the total gaseous exchange collected for 5 hours of work and recovery was practically the same as the resting quotient. The rise in R.Q. therefore appears to be indicative of an acid-base change or a "blowing off" of CO_2 rather than of carbohydrate oxidation. The absence of sugar combustion could hardly be attributed to a lack of an available carbohydrate supply in the body, for in every case except after the second injection in Dog 63 (Table V) the epinephrine produced a large outpouring of extra glucose in the urine, and in the other series the tissue analysis showed that the muscle and liver glycogen was not exhausted. These data are considered as additional evidence that the energy for muscular contraction and recovery in the diabetic animal may be derived from the oxidation of fat.

SUMMARY

Exercise and recovery have been studied in dogs fasted for 3 to 13 days after pancreatectomy and in some of the same dogs under similar conditions before operation.

In one series of experiments, performed under amytal anesthesia, contraction of the leg muscles was caused by stimulation with an induced electric current. The respiratory metabolism, blood lactic acid, and blood sugar changes were determined during a pre-work resting period, 1 hour of work, and about 4 hours of recovery. The elevation in R.Q. during exercise was accompanied by a rise in blood lactic acid. After the work ceased the R.Q. fell below the resting level and the blood lactic acid concentration returned to normal. In the later post-work periods the quotients came back to the normal diabetic values.

In another series muscular contraction was stimulated by the injection of large doses of epinephrine (0.25 to 0.6 mg. per kilo). During the 1st hour of observation when the animals were active there was an average rise in R.Q. of about 0.06. During the later hours, when the dogs were quiet, a compensatory fall in quotient was found such that the R.Q. for the entire 5 hours was practically the same as that of the preceding basal period.

A comparison of the calculated excess heats of the post-work periods suggests a slightly slower recovery process in the diabetic than in the normal fasting dog.

Analyses of the tissues of the diabetic dogs for glycogen, sugar, and lactic acid showed no particular differences before or after work. The carbohydrate content of the cardiac muscle was high, that of the leg muscles and liver was not exhausted at death. The diaphragm resembled the striated muscle rather than the heart in its glycogen content.

These data indicate that the rise in R.Q. was due to changes in the CO_2 equilibrium in the body rather than to carbohydrate oxidation and favor the theory that fat is the fuel of exercise in the depancreatized dog.

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MUSCLE CREATINE IN NUTRITIONAL MUSCULAR DYSTROPHY OF THE RABBIT*

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The creatine content of normal muscle is, within the rather wide limits of variation, constant for each species (1), although not all types of muscle in the same animal have the same creatine content. The skeletal muscles are known to be relatively rich in this substance, the white form exceeding the red. Cardiac muscle contains roughly about one-half as much as the mixed skeletal and considerably more than smooth muscle, which contains no more than the visceral organs.

Exercise apparently does not affect the creatine concentration of striated muscle, nor does autolysis, even after 3 days (2). An increase has been observed during the early stages of inanition (3) and in avitaminosis, particularly in scurvy (4, 5), and may be experimentally induced, as many workers have shown, by the administration of creatine. On the other hand, a decrease in creatine concentration has been noted in the final stages of inanition (6), and in human muscle under pathological conditions involving extreme emaciation (7–9), or muscle disorders, such as myasthenia gravis (10) and myositis fibrosa (11). Bodansky, Schwab, and Brindley (11) were able to show a definite correlation between the creatine content in the muscle and the extent of pathological lesions.

In a previous paper (12) some diets were described, upon which guinea pigs and rabbits were found to develop an extreme degeneration of the voluntary muscles without obvious lesions in the other organs. It was hoped that an investigation of the chemistry of these muscles would contribute not only to an understanding of the disease, but also to the development of a chemical means of diagnosis. Such a study was initiated with creatine determinations. In this paper are reported the results of analyses for creatine, total nitrogen, moisture, and fat, carried out on 62 muscles obtained from fifteen rabbits in various stages of nutritional muscular dystrophy and on twenty-eight normal muscles from five controls. Analyses of cardiac muscle, which remains normal in animals with severely degenerated skeletal muscle, have been included, as well as some determinations carried out on brain.

<div align="center">EXPERIMENTAL</div>

The tissues for analysis were taken from adult rabbits that had been fed Diet 11 or 13,[1] with and without natural food supplements, in experiments planned for the prevention of the disorder and to be reported elsewhere. The animal was usually killed by a blow during the last stages of prostration due to muscular weakness, and immediately sampled, although in a few instances material was taken some hours after death. Normal

[1] Diets 11 and 13 are as follows:

<div align="center">*Diet 13*</div>

		parts
Rolled oats (Quaker)		355
Wheat bran (Pillsbury)		180
Casein (Merck's technical)		75
Lard		80
Cod liver oil (Mead Johnson)		10
NaCl		10
CaCO$_3$		15

10 gm. of ferric chloride, u.s.p. lump, were taken up in about 125 cc. of ether and a little water, and the solution poured over the above ingredients. The mass was shaken in a closed container and allowed to stand for about half an hour. The contents were emptied upon a tray and the ether allowed to evaporate, usually overnight. Finally there was added skim milk powder (Merrell-Soule), 275 parts.

Diet 11 was similar to Diet 13 except that the treatment with ethereal ferric chloride was omitted.

tissue was obtained from adult rabbits that had been maintained on a stock diet of oats, hay, and greens.

The muscles were analyzed separately. White muscles were represented by the gluteus, gastrocnemius, and triceps brachii (caput longum); red muscle by the semitendinosus, soleus, and triceps brachii (caput mediale). The muscles were freed as far as possible from connective tissue and, from each pair, samples were taken for histological study and for the determination of moisture, creatine, total nitrogen, and fat, in so far as quantity permitted.

Methods of Analysis

The moisture determinations were carried out in small glass-stoppered weighing bottles with samples weighing 0.1 to 0.3 gm. Constant weight was found to be attained after 3 hours at 100°, but for routine determinations a 20 hour period was adopted. Duplicate estimations gave an average difference of 3 per cent.

The creatine was estimated as creatinine by the Rose, Helmer, and Chanutin (13) modification of the Folin (14) method. Samples weighing from 0.2 to 1.5 gm. depending upon the amount of tissue available, were weighed in glass-stoppered weighing bottles. It was found convenient to prepare five standards containing from 0.1 to 1.0 mg. of creatinine. There was an average difference of 7 per cent between determinations carried out in duplicate. An additional source of error was the discrepancy, which averaged 3 per cent, encountered when two analysts made the colorimetric comparison.

Total nitrogen was determined according to the Arnold-Gunning modification (15) of the Kjeldahl method, 1 to 2 gm. of fresh tissue being used.

Leathes' (16) method was used for the estimation of fat.

Results

The results of the analyses for creatine of tissue from normal rabbits and those with nutritional muscular dystrophy are plotted in Chart 1. The muscles were arbitrarily graded from + to 4+, according to the severity of the lesions. This grading, however, takes no account of the variation in the pathological pictures, which depend upon the acuteness or chronicity of the lesions. Thus, a 4+ lesion may refer to an extreme hyaline necrosis affect-

ing almost 100 per cent of the fibers, or it may refer to an extreme loss of fibers with fibrous or lipomatous replacement. The creatine values for each kind of tissue have been further described by including in Chart 1 the mean, the standard deviation, and the coefficient of variation, together with their respective probable errors.

WHITE MUSCLE					RED MUSCLE				OTHER TISSUES		
Extent of Muscle Dystrophy					Extent of Muscle Dystrophy				Heart	Brain	Connective Tissue
NORMAL	+	+ +	+ + +	+ + + +	NORMAL	+	+ +	+ + +			

MG CREATINE PER 100 GM. FRESH TISSUE

600 — 500 — 400 — 300 — 200 — 100 — 0

	WHITE — NORMAL	WHITE + + + +	RED — NORMAL		Heart	Brain
NUMBER	16	····20	····13		····16	····16
MEAN	463	174	314		188	110
	±51	±95	±71		±44	±23
STD.DEV.	±30	164	138		±26	±14
	±36	±68	±50		±30	±17
COEF.OF VAR.	7	37	12		14	13
	±08	±42	±16		±17	±16
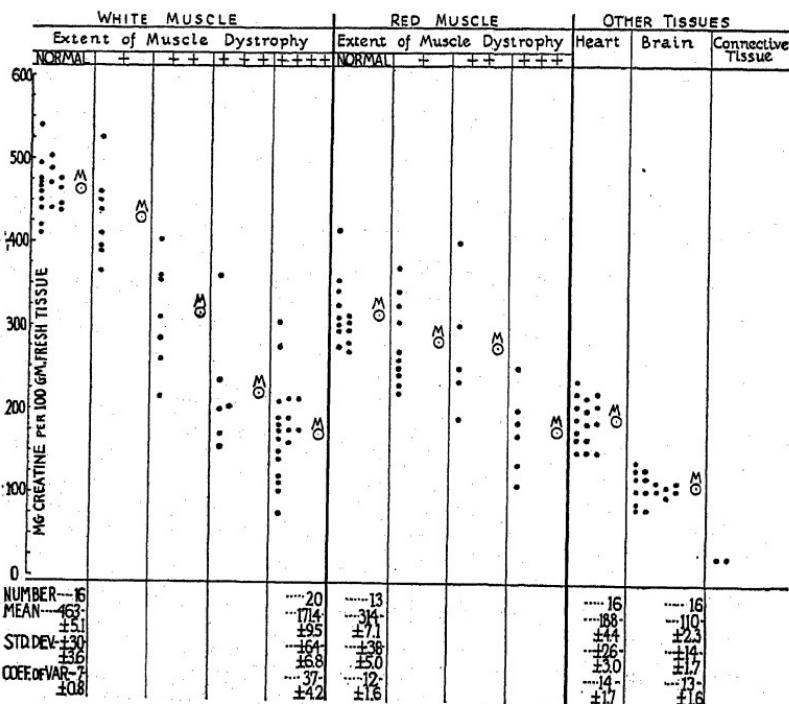
CHART 1. Illustrating creatine concentration of tissue from normal adult rabbits and those with nutritional muscular dystrophy. M shows the position of the mean value.

The values obtained for creatine in normal tissue agree with those given in the literature (1). Degenerated muscle differs markedly from normal muscle in its creatine concentration, for in the case of the most severe lesions found, the values for creatine lie distinctly outside the range of variation for normal muscle. It will be noticed that white muscle loses more creatine than red, and that in both kinds of severely dystrophic muscle, the creatine

concentration, although showing greater variation, is reduced to that normally found in myocardium.

Although the skeletal muscle of rabbits with nutritional muscular dystrophy was characterized by a low creatine value, the heart

WHITE MUSCLE			RED MUSCLE		HEART	BRAIN
mg. Creatine per 100 g.Tissue			C. per 100 g. Tissue			
Above 400	250– 400	Below 250	Above 250	Below 250		
21		14	17		14	16
75.7±0.2		79.5±0.5	75.8±0.2		77.8±0.4	79.2±0.2
±1.4±0.1		±2.7±0.3	±1.4±0.1		±1.9±0.3	±1.4±0.1
1.8±0.2		3.4±0.4	1.8±0.2		2.4±0.3	1.8±0.2

Number / Mean / Std. Dev. / Coeff. of Var.

Percent Moisture vertical axis: 82.0, 80.0, 78.0, 76.0, 74.0, 72.0
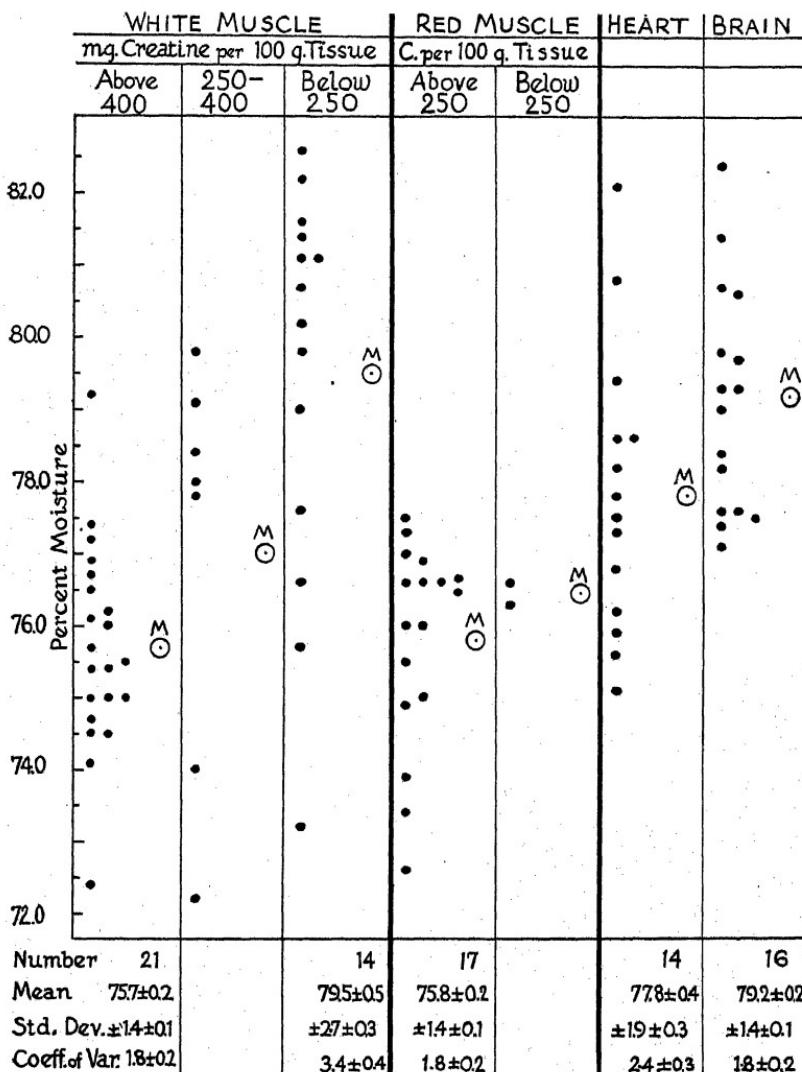
CHART 2. Illustrating moisture content of tissue from normal adult rabbits and those with nutritional muscular dystrophy. *M* shows the position of the mean value.

and brain of such animals upon analysis gave the same values as heart and brain from normal animals. All results obtained are therefore grouped together in Chart 1.

The results of analyses for moisture are graphically illustrated in Chart 2. Only muscles with normal fat content are included.

WHITE MUSCLE			RED MUSCLE		HEART	BRAIN
mg. Creatine per 100 g. Tissue			C. per 100 g. Tissue			
Above 400	250-400	Below 250	Above 250	Below 250		

(Percent Total Nitrogen plotted on vertical axis: 4.20, 3.80, 3.40, 3.00, 2.60, 2.20, 1.80, 1.40)

	Number	Mean	Std. Dev.	Coeff. of Var.
White Muscle	21	3.5±0.04	±0.3±0.03	8±0.83
	19	2.6±0.04	±0.3±0.03	12±1.32
Heart	13	2.7±0.04	±0.2±0.03	8±1.1
Brain	13	1.8±0.04	±0.2±0.03	11±1.6
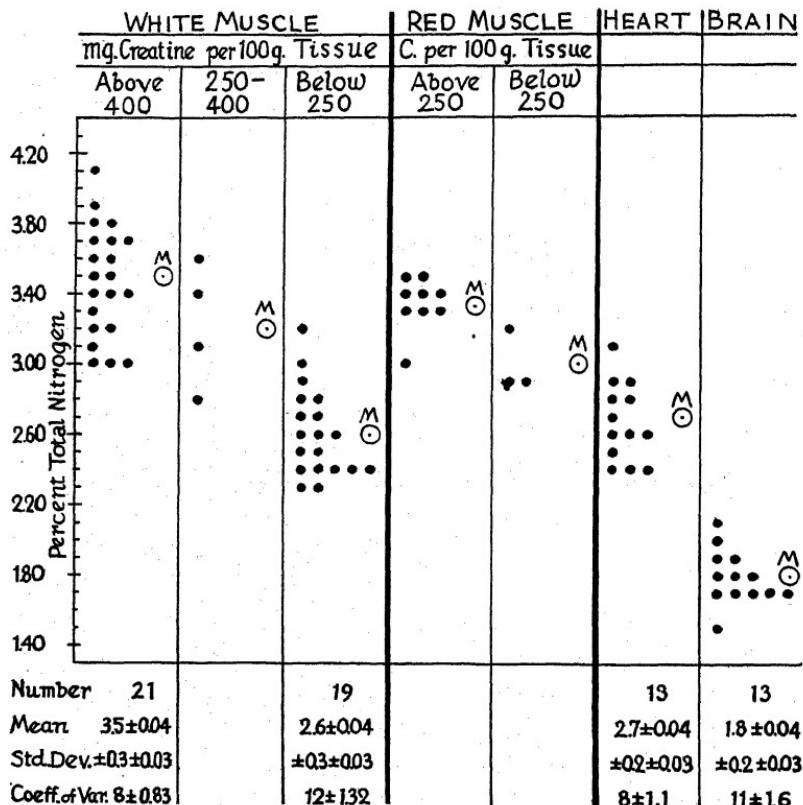
CHART 3. Illustrating total nitrogen concentration of tissue from normal adult rabbits and those with nutritional muscular dystrophy. *M* shows the position of the mean value.

Instead of classification according to the pathological picture, a criterion which is not quantitative, the muscles are grouped with respect to creatine content. Three arbitrary ranges were chosen for white muscle, two for red. It is apparent from Chart 2 that in muscle with low creatine content there is a definite increase in moisture.

That the increase in moisture does not account for the low crea-
tine content of degenerated muscle was proved by a recalculation
of the creatine values to a dry weight basis.   The difference in
creatine concentration between normal and degenerated muscle
remains significant.

WHITE MUSCLE			RED MUSCLE		HEART	BRAIN
mgCreatine per 100 g. Tissue			C. per 100 g. Tissue			
Above 400	250-400	Below 250	Above 250	Below 250		
Number 21		19			13	19
Mean 4.3±0.07		2.1±0.08			2.2±0.07	2.0±0.06
Std.Dev.±0.5±0.05		±0.5±0.05			±0.4±0.05	±0.3±0.04
Coeff.of Var.12±1.25		24±2.63			18±2.5	15±2.1
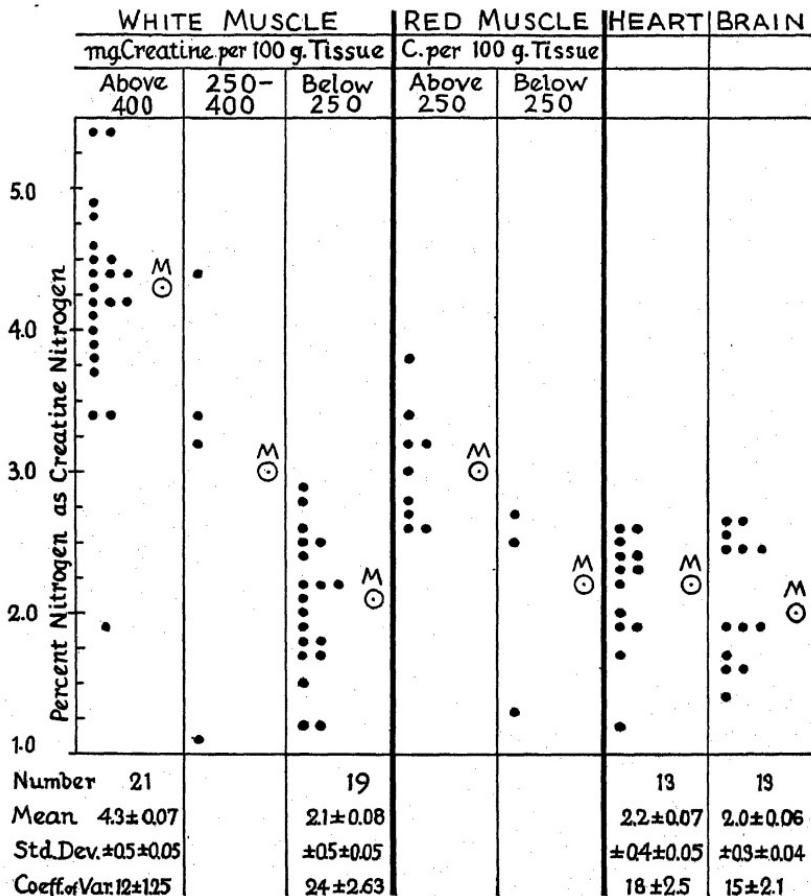
CHART 4. Illustrating the proportion of nitrogen present as creatine
in tissue from normal adult rabbits and those with nutritional muscular
dystrophy. M shows the position of the mean value.

In Chart 3 are plotted the results of the analyses for total nitro-
gen, the muscles being classified according to the creatine content
as in Chart 2.   The decrease in nitrogen content of muscle low in
creatine is apparent.

In normal white muscle 4 per cent of the total nitrogen is derived from creatine, in normal red muscle, 3 per cent, and in the heart and brain 2 per cent, as shown in Chart 4. The values for the white muscle and heart are in agreement with the values obtained by Folin and Buckman (17). It is also apparent that in the muscle, both white and red, with low creatine the nitrogen present in this form has fallen to 2 per cent. In a series of human muscle determinations by Denis (9), the lowest ratio was 2.1 per cent, occurring in Graves' disease.

TABLE I

Results of Analyses of Tissue from Rabbit 29 with Severe Nutritional Muscular Dystrophy

Kind of tissue	Moisture	Fat	Creatine per 100 gm.		Total N	N as creatine N	Extent of pathological lesion
			Fresh tissue	Dry tissue			
	per cent	*per cent*	*mg.*	*mg.*	*per cent*	*per cent*	
White muscle							
Gluteus..............	76.2	5.3	210	900	2.4	2.8	++++
Gastrocnemius......	73.6		90	340	2.5	1.2	++++
Triceps.............	72.9	7.5	120	430	2.5	1.5	++++
Red muscle							
Semitendinosus.....	77.4		220	970			+
Soleus..............	79.5		110	550			+++
Triceps.............	77.4		230	1000	2.9	2.5	+
Heart................	77.5		220	980	2.8	2.5	Normal
Brain................	82.4		130	750	1.8	2.3	"

Table I, containing the results of analyses carried out on tissue from Rabbit 29 with characteristic muscular dystrophy, presents the type of data from which the charts were formulated. The creatine content, although extremely variable, is seen to be roughly related to the extent of pathological disorder in the muscle. Table I also illustrates the fact, mentioned in the previous paper, that not all skeletal muscles are uniformly affected. White muscle is apparently more extensively injured than red, for in animals with severely degenerated white muscle the red was frequently observed to be only slightly abnormal. In the fifteen rabbits studied, 4+ lesions were never found in red muscle. Of the three white muscles chosen for study, none showed any tendency to-

wards greater degeneration. The same applies to the three red muscles.

The results of the analyses for fat are not included in the charts because of the extreme variability of fat in degenerated muscle. Table I includes fat determinations of muscle from Rabbit 29, in which the fat content was from 4 to 5 times as great as normal. Table II gives, in addition, the results of analyses of muscle high in fat from two other rabbits, as well as from a typical control. The creatine content of severely degenerated muscle is seen to be similar, regardless of the amount of fat present. Not only creatine but also total nitrogen of these muscles was found to be independent of the fat concentration. The extent of fatty infiltration in

TABLE II

Analyses of Some Typical Dystrophic White Muscle High in Fat, and Normal Controls

Rabbit No.	Kind of muscle	Fat	Moisture	Creatine per 100 gm. fresh muscle	Total N	N as creatine N	Extent of pathological lesion
		per cent	*per cent*	*mg.*	*per cent*	*per cent*	
61	Gluteus	0.7	76.1	490	3.8	4.2	Normal
	Triceps	1.1	76.5	470	3.3	4.6	"
25	Gluteus	22.0	66.7	200	2.9	2.2	+++
	Triceps	22.2	61.2	160	2.7	1.9	++++
56	Gluteus	48.0	39.6	230	2.6	2.9	+++
	Triceps	34.5	50.1	190	2.3	2.6	++++

the muscle depends, in general, upon the length of time of survival of the animal upon the diet.

Although the extent of lipomatosis in the degenerated muscle could be determined chemically, and its lack of influence upon the creatine level established, the effect of fibrous replacement could be only indirectly studied. Unfortunately there is no adequate quantitative chemical test for connective or fibrous tissue. Subcutaneous loose connective tissue was carefully teased out, with as little drying as possible, from two normal rabbits and analyzed for moisture, creatine, and total nitrogen. The results of the analyses are given in Table III.

The creatine values are low, as are the figures for total nitrogen. The per cent of nitrogen in the form of creatine, however, approximates that for normal muscle.

In rabbits with nutritional muscular dystrophy there is usually marked wasting of muscle tissue.   The extent of the loss in weight is apparent from Table IV, which contains the weights of indi-

TABLE III

Differences between Connective Tissue and Muscle in Normal Rabbits with Respect to Moisture, Creatine, and Total Nitrogen

Rabbit No.	Kind of tissue	Moisture	Creatine per 100 gm.		Total N	N as creatine N
			Fresh tissue	Dry tissue		
		per cent	mg.	mg.	per cent	per cent
63	Gluteus muscle	76.9	410	1770	3.50	3.7
	Heart	78.6	150	710	2.60	1.8
	Connective tissue	87.7	26	210	0.26	3.1
64	Gluteus muscle	75.4	470	1910	3.40	4.4
	Heart	79.4	150	728	2.60	1.8
	Connective tissue	80.0	24	120	0.19	4.2

TABLE IV

Showing Loss in Weight of Individual Muscles in Rabbits with Dystrophy

Rabbit No.	Weight of rabbit		Kind of muscle	Weight of muscle	Per cent of body weight at death	Creatine per 100 gm. muscle	Extent of pathological lesion
	3 wks. before death	At death					
	kg.	kg.		gm.		mg.	
61	3.75	3.65	Gastrocnemius	15.68	0.42	490	Normal
			Triceps (white)	13.96	0.37	470	"
			Soleus	1.48	0.04	320	"
			Triceps (red)	2.46	0.06	300	"
42	3.95	3.05	Gastrocnemius	10.58	0.35	400	++
			Triceps (white)	8.56	0.29	440	Normal
			Soleus	0.70	0.02	400	++
			Triceps (red)	1.89	0.05	370	+
56	2.75	2.00	Gastrocnemius	5.43	0.27	270	++++
			Triceps (white)	3.79	0.19	190	++++
			Soleus	0.82	0.04	270	+
			Triceps (red)	1.37	0.06	250	+++

vidual normal muscles and of some that were dissected from rabbits in different stages of the disease.   Although some muscles retain their relative weights, others may lose from 20 to 50 per cent during the course of the dystrophy.

The creatine concentration of skeletal muscle from rabbits in the last stages of nutritional muscular dystrophy is significantly lower than that of normal tissue. The decrease is roughly proportional to the amount of pathological degeneration. It is independent of the extent of fatty or fibrous infiltration, but how much of the decrease in creatine is due to the reduction in number of normal muscle fibers, how much to the lowered creatine content of abnormal ones, cannot yet be stated. It is interesting in this connection that Williams and Dyke (10) found a reduction of the muscle creatine in myasthenia gravis, a disease in which there is no appreciable degeneration or numerical loss of muscle fibers. Furthermore, low creatine values have been found by us in muscles showing only necrosis without fibrosis or fat replacement. There is no way of determining the amount of normal muscle cells in the tissue by known chemical means, and although the study of serial sections might yield an approximation of the relative amounts of normal fibers, abnormal ones, and connective tissue present, the results would not be quantitative.

Fasting is a factor to be considered in the explanation of low creatine values in muscle, because the final stages of nutritional muscular dystrophy are usually accompanied by at least partial inanition. However, this period is of short duration and represents the early period of fasting of Myers and Fine (6) who found that the creatine content of muscle was increased at this time. Some of the rabbits studied were sacrificed before there had been any refusal of food, but no essential differences were noted.

It is interesting that in this series of rabbit muscle, the creatine concentration fell to the level at which it is normally present in the heart, not to the level characteristic of the visceral organs. In the human muscle series by Bodansky, Schwab, and Brindley (11) the lowest value for creatine obtained likewise equaled that found in the heart. This observation may be merely a coincidence, or it might indicate that creatine is bound in more than one way in striated muscle. A study of these muscles for phosphocreatine (18) and other fractions of phosphorus, some of which have been shown by several workers to be related to creatine or nitrogen concentration, is in progress and it is planned to correlate

the values so obtained with the creatine and nitrogen of the same muscle.

On account of the great variation of creatine in normal muscle and in each classified group of degenerated muscle as shown in Chart 1, the creatine content of muscle indicates with certainty the presence of muscular dystrophy, but only roughly its severity. Since muscle with slight lesions retains approximately the normal amounts, the method is not particularly useful in curative experiments, in which at present the onset of the disease can be determined only by a study of the pathology of biopsy material. As creatinuria usually accompanies conditions of muscular disorder, the creatine metabolism of rabbits on Diet 11 is being studied as a possible means of establishing the inception of the disease.

SUMMARY

In nutritional muscular dystrophy of the rabbit, there is an absolute as well as relative loss of creatine in the skeletal muscle without any demonstrable loss in either heart or brain. Both white muscle (which normally contains 420 to 500 mg. of creatine per 100 gm. of fresh tissue) and red muscle (260 to 360 mg.) contain, when in the last stages of degeneration, 110 to 250 mg.; this is the level at which creatine is normally present in the heart. In both types of muscle, the proportion of nitrogen present as creatine falls to 2 per cent, the ratio characteristic for heart and brain, in contrast to the normal ratios for white and red muscle, 4 and 3 per cent respectively.

The creatine concentration of degenerated muscle was found to be independent of the fat content, which varied from 0.5 to 48.0 per cent.

The moisture content of degenerated muscle with normal fat content is higher than that of normal muscle.

In this disease, white muscle shows not only a greater reduction in creatine than does red muscle, but displays pathological lesions of greater severity.

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CHEMICAL STRUCTURE AND OPTICAL ROTATION

III. THE CONFIGURATIONAL RELATIONSHIP OF DISUBSTITUTED PROPIONIC ACIDS CONTAINING A CYCLOHEXYL GROUP

ALSO, A CORRECTION TO THE PAPER ON THE CONFIGURATIONAL RELATIONSHIP OF DISUBSTITUTED PROPIONIC ACIDS CONTAINING A PHENYL GROUP

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New York*)

The present communication deals with the direction of rotation of the individual members of the homologous series of the derivatives of the disubstituted (in position (3)) propionic acids containing a cyclohexyl group. The members of this group of substances were prepared in two ways: first, by catalytic reduction of the corresponding phenyl derivatives and second, by direct synthesis.

However, before discussing this relationship, we have to make a correction regarding the results previously published dealing with the direction of rotation of the individual members of the disubstituted propionic acids containing a phenyl group. In an earlier article,[1] the sign of rotation of 3-propyl-3-phenylacetic acid should be changed from minus to *plus* and the signs of the derivatives should be changed in accordance with Table I of this paper.

The relationship of 3-ethyl-3-phenylpropionic and 3-propyl-3-phenylpropionic acids was established by reactions shown in I.

In the previous communication[1] it was reported that ethylpropylphenylmethane obtained from either of the two acids was levorotatory which then naturally led to the conclusion that the two acids were enantiomorphous. It is now found that the levorotation of that hydrocarbon was due to the partial unsaturation which occurred in the process of reduction of the bromide. The reduction of the hydrocarbon with hydrogen in the presence of

[1] Levene, P. A., and Marker, R. E., *J. Biol. Chem.*, **93**, 749 (1931).

$$\underset{\underset{C_6H_5}{|}}{\overset{\overset{C_2H_5}{|}}{HC}}\text{—}CH_2COOH \longrightarrow \underset{\underset{C_6H_5}{|}}{\overset{\overset{C_2H_5}{|}}{HC}}\text{—}CH_2CH_2Br \longrightarrow \underset{\underset{C_6H_5}{|}}{\overset{\overset{C_2H_5}{|}}{HC}}\text{—}C_3H_7$$

Levo   Levo   Levo

$$\underset{\underset{C_6H_5}{|}}{\overset{\overset{C_3H_7\ (n)}{|}}{HC}}\text{—}CH_2COOH \longrightarrow \underset{\underset{C_6H_5}{|}}{\overset{\overset{C_3H_7\ (n)}{|}}{HC}}\text{—}CH_2CH_2Br \longrightarrow \underset{\underset{C_6H_5}{|}}{\overset{\overset{C_3H_7}{|}}{HC}}\text{—}C_2H_5$$

Levo   Levo   Dextro
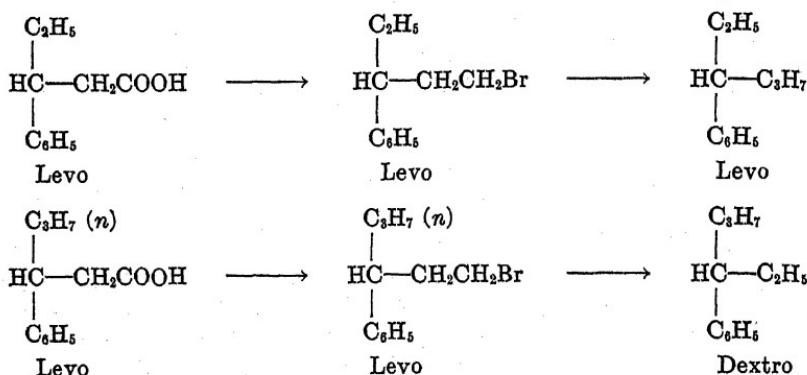
colloidal palladium at atmospheric pressure brought about a change in the direction of rotation of the hydrocarbon. Doubt as to the correctness of the earlier findings was aroused in course of the work on the cyclohexyl derivatives; hence, all the hydrocarbons of the phenyl series were reexamined for their state of saturation but only those derived from 3-propyl-3-phenylpropionic acid were found partially unsaturated. In order further to verify the present conclusion, 3-butyl-3-phenylpropionic acid was prepared and its relationship to 3-ethyl-3-phenylpropionic acid was established by the set of reactions in II.

$$\underset{\underset{C_6H_5}{|}}{\overset{\overset{C_2H_5}{|}}{HC}}\text{—}CH_2COOH \longrightarrow \underset{\underset{C_6H_5}{|}}{\overset{\overset{C_2H_5}{|}}{HC}}\text{—}CH_2CH_2Br \longrightarrow \underset{\underset{C_6H_5}{|}}{\overset{\overset{C_2H_5}{|}}{HC}}\text{—}C_4H_9$$

Levo   Levo   Levo

$$\underset{\underset{C_6H_5}{|}}{\overset{\overset{C_4H_9\ (n)}{|}}{HC}}\text{—}CH_2COOH \longrightarrow \underset{\underset{C_6H_5}{|}}{\overset{\overset{C_4H_9\ (n)}{|}}{HC}}\text{—}CH_2CH_2Br \longrightarrow \underset{\underset{C_6H_5}{|}}{\overset{\overset{C_4H_9}{|}}{HC}}\text{—}C_2H_5$$

Levo   Levo   Dextro
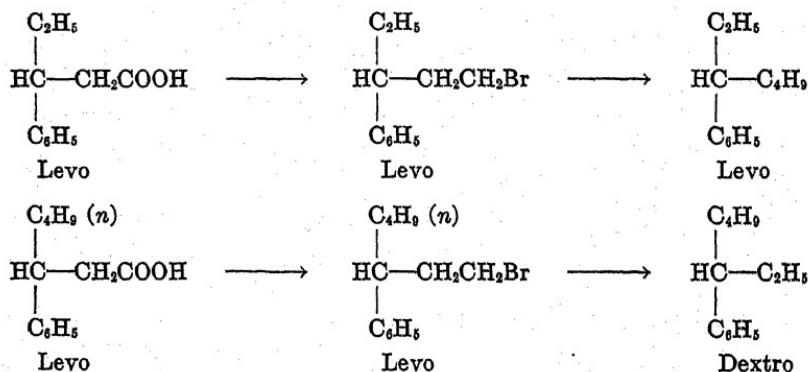
II

Thus, it may be concluded that all members of the homologous series of the 3-alkyl-3-phenylpropionic acid series rotate in the

TABLE I*

Configurational Relationship of Phenyl Compounds, $[M]_D^{25}$ (Not Maximum Rotations)

CH_3 $HC{-}CH_2COOH$ C_6H_5 +20.3	CH_3 $HC{-}CH_2COOC_2H_5$ C_6H_5 +14.2	CH_3 $HC{-}CH_2CH_2OH$ C_6H_5 +13.7	CH_3 $HC{-}CH_2CH_2Br$ C_6H_5 +39.5	CH_3 $HC{-}CH_3$ C_6H_5 0	CH_3 $HC{-}C_2H_5$ C_6H_5 +9.1	CH_3 $HC{-}C_3H_7(n)$ C_6H_5 	CH_3 $HC{-}C_4H_9(n)$ C_6H_5 +9.9
C_2H_5 $HC{-}CH_2COOH$ C_6H_5 +26.9†	C_2H_5 $HC{-}CH_2COOC_2H_5$ C_6H_5 +15.3	C_2H_5 $HC{-}CH_2CH_2OH$ C_6H_5 +7.5	C_2H_5 $HC{-}CH_2CH_2Br$ C_6H_5 +50.5	C_2H_5 $HC{-}CH_3$ C_6H_5 −2.6	C_2H_5 $HC{-}C_2H_5$ C_6H_5 0	C_2H_5 $HC{-}C_3H_7(n)$ C_6H_5 +1.9	C_2H_5 $HC{-}C_4H_9(n)$ C_6H_5 +3.5
$C_3H_7(n)$ $HC{-}CH_2COOH$ C_6H_5 +11.4	$C_3H_7(n)$ $HC{-}CH_2COOC_2H_5$ C_6H_5 +7.6	$C_3H_7(n)$ $HC{-}CH_2CH_2OH$ C_6H_5 +3.2	$C_3H_7(n)$ $HC{-}CH_2CH_2Br$ C_6H_5 +26.4	$C_3H_7(n)$ $HC{-}CH_3$ C_6H_5 	$C_3H_7(n)$ $HC{-}C_2H_5$ C_6H_5 −0.9	$C_3H_7(n)$ $HC{-}C_3H_7(n)$ C_6H_5 0	$C_3H_7(n)$ $HC{-}C_4H_9(n)$ C_6H_5 +0.4
$C_4H_9(n)$ $HC{-}CH_2COOH$ C_6H_5 +10.3	$C_4H_9(n)$ $HC{-}CH_2COOC_2H_5$ C_6H_5 +7.0	$C_4H_9(n)$ $HC{-}CH_2CH_2OH$ C_6H_5 +2.6	$C_4H_9(n)$ $HC{-}CH_2CH_2Br$ C_6H_5 +26.5	$C_4H_9(n)$ $HC{-}CH_3$ C_6H_5 −9.9‡	$C_4H_9(n)$ $HC{-}C_2H_5$ C_6H_5 −1.7	$C_4H_9(n)$ $HC{-}C_3H_7(n)$ C_6H_5 −0.4‡	$C_4H_9(n)$ $HC{-}C_4H_9(n)$ C_6H_5 0

* The sign of rotation of the values in Tables I to VIII of the previous paper (*J. Biol. Chem.*, **93**, 750 (1931)) should be changed to conform to the above values.

† In benzene.

‡ Enantiomorphous.

same direction. In order to avoid confusion, the direction of rotation of all the derivatives of 3-alkyl-3-phenylpropionic acids are summarized in Table I.[2]

Returning now to the derivatives of the corresponding cyclohexyl series, we found that the hydrogenation of the 3-methyl-3-

TABLE II—*Configurationally Related Cyclohexyl Compounds (Not Maximum Rotations, $[M]_D^{25}$, by R*

CH_3 $HC-CH_2COOH$ C_6H_5 +13.6	−1.3 CH_3 $HC-CH_2COOH$ C_6H_{11} −0.4	−2.9 CH_3 $HC-CH_2COOC_2H_5$ C_6H_{11}	−4.2 CH_3 $HC-CH_2CH_2OH$ C_6H_{11} −4.2	−15.2 CH_4 $HC-CH_2CH_2Br$ C_6H_{11} −14
C_2H_5 $HC-CH_2COOH$ C_6H_5 +25.3‡	+2.5 C_2H_5 $HC-CH_2COOH$ C_6H_{11} +2.7	+1.7 C_2H_5 $HC-CH_2COOC_2H_5$ C_6H_{11}	−3.4 C_2H_5 $HC-CH_2CH_2OH$ C_6H_{11} −3.8	C_2H_5 $HC-CH_2CH_2Br$ C_6H_{11} −4
$C_3H_7(n)$ $HC-CH_2COOH$ C_6H_5 +11.8	+4.0 $C_3H_7(n)$ $HC-CH_2COOH$ C_6H_{11} +1.9	+3.9 $C_3H_7(n)$ $HC-CH_2COOC_2H_5$ C_6H_{11}	−1.4 $C_3H_7(n)$ $HC-CH_2CH_2OH$ C_6H_{11} −0.4	−4 $C_3H_7(n)$ $HC-CH_2CH_2Br$ C_6H_{11} −5
$C_4H_9(n)$ $HC-CH_2COOH$ C_6H_5 +47.7	$C_4H_9(n)$ $HC-CH_2COOH$ C_6H_{11} +9.7			

* By direct synthesis.

† By reduction of corresponding phenyl derivative.

‡ In benzene.

phenylpropionic acid to the corresponding cyclohexyl derivative resulted in a change of the direction of rotation. The hydrogenation of the higher members did not result in a change of direction

[2] We wish to mention that there recently has come to our notice a publication by Cohen, Marshall, and Woodman (*J. Chem. Soc.*, **107**, 887 (1915)) in which an account is given of the resolution of β-phenylpropyl alcohol and of its conversion into the bromide. The two substances in homogeneous state rotated in the same direction. The same two sub-

of rotation. On the other hand, all the carbinols, the halides, and hydrocarbons of the cyclohexyl series rotated in a direction opposite to that of the corresponding members of the phenyl series (see Table II).

Thus, the first member of the homologous series of 3-methyl-3-

eduction of Corresponding Phenyl Derivative and by Direct Synthesis

0	−1.5	−3.8		*
CH_3	CH_3	CH_3	CH_3	
$HC-CH_3$	$HC-C_2H_5$	$HC-C_3H_7(n)$	$HC-C_4H_9(n)$	
C_6H_{11}	C_6H_{11}	C_6H_{11}	C_6H_{11}	
0	−1.1		−4.5	†
	0			*
C_2H_5	C_2H_5	C_2H_5	C_2H_5	
$HC-CH_3$	$HC-C_2H_5$	$HC-C_3H_7(n)$	$HC-C_4H_9(n)$	
C_6H_{11}	C_6H_{11}	C_6H_{11}	C_6H_{11}	
Dextro	0	−1.5	−2.5	†
	+1.0	0		*
$C_3H_7(n)$	$C_3H_7(n)$	$C_3H_7(n)$	$C_3H_7(n)$	
$HC-CH_3$	$HC-C_2H_5$	$HC-C_3H_7(n)$	$HC-C_4H_9(n)$	
C_6H_{11}	C_6H_{11}	C_6H_{11}	C_6H_{11}	
Dextro		0	−0.5	†
	$C_4H_9(n)$			*
	$HC-C_2H_5$			
	C_6H_{11}			
	+5.6			†

cyclohexylpropionic acids rotates in a direction opposite to that of the other members, resembling in this respect the behavior of the first member of the 3-methyl-3-ethylpropionic acid series.

stances were prepared by Levene, Mikeska, and Passoth (*J. Biol. Chem.*, **88**, 27 (1930)) by a different method and were found to rotate in opposite directions. Because of this discrepancy, the topic is in need of reinvestigation. Work in this direction is in progress.

The direction of rotation of the trisubstituted methanes containing a cyclohexyl group has been found again to be determined by the position of the heavier alkyl group, thus following the same rule as in the case of the acyclic normal secondary carbinols, or of the other hydrocarbons studied up to the present.

$$[M]_D^{25}$$
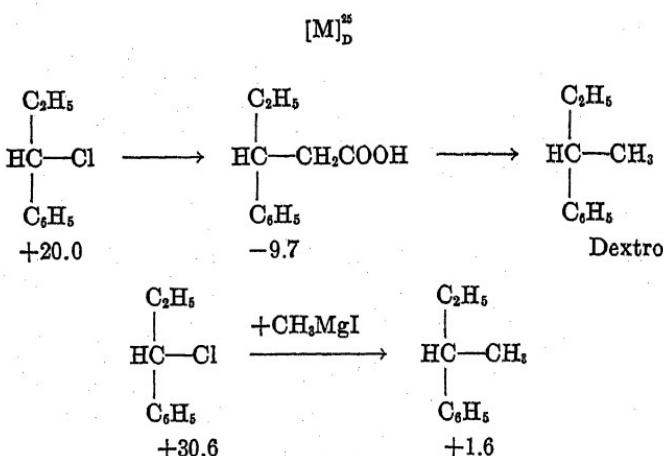
III

Incidentally, it may be mentioned in this place that in the case of ethylphenylchloromethane the substitution of the chlorine atom either by the malonic ester residue or by a methyl group through the Grignard reaction is accomplished in the same stereochemical sense; that is, in both cases either with or without Walden inversion.

<div style="text-align:center">EXPERIMENTAL</div>

2-Cyclohexylbutyric Acid-4 (by Catalytic Reduction)—20 gm. of 2-phenylbutyric acid-4, $[M]_D^{25} = +13.57°$ (homogeneous), were mixed with 50 gm. of glacial acetic acid and 1 gm. of platinic oxide catalyst. The acid was reduced by shaking with hydrogen under 30 pounds pressure. Reduction was complete in 8 hours. When absorption of hydrogen ceased, 1 gm. of additional platinic oxide was added and shaking continued for 16 hours longer. There was no further absorption of hydrogen during this last shaking. The product was filtered and fractionated. The reduced acid was

converted into the sodium salt in water and extracted with ether to remove impurities. This solution was acidified with hydrochloric acid, extracted with ether, and distilled. B. p. 145° at 4 mm. Yield 14 gm. D $\frac{24}{4}$ = 1.018.

$$[\alpha]_D^{24} = \frac{-0.27°}{1 \times 1.018} = -0.26°; [M]_D^{24} = -0.44° \text{ (homogeneous)}$$

4.400 mg. substance: 11.465 mg. CO_2 and 4.270 mg. H_2O
$C_{10}H_{18}O_2$. Calculated. C 70.5, H 10.6
170.14   Found.   " 71.0, " 10.8

Dextro-3-Cyclohexylbutanol-1 (by Catalytic Reduction)—15.6 gm. of 3-phenylbutanol-1, $[\alpha]_D^{25} = -9.15°$ (homogeneous), were dissolved in 25 cc. of glacial acetic acid and reduced by shaking with 1 gm. of platinic oxide catalyst under hydrogen at 30 pounds pressure. The reduction was complete in 6 hours. In order to insure complete reduction, at the end of this period 0.5 gm. additional catalyst was added and shaking continued under hydrogen for 6 hours more. The acetic acid solution was filtered and the acid neutralized with sodium carbonate solution. The carbinol was extracted with ether, dried with sodium sulfate, then distilled. B. p. 128° at 15 mm. Yield 15 gm. $n_D^{25} = 1.4701$; D $\frac{22}{4}$ = 0.919.

$$[\alpha]_D^{22} = \frac{+2.49°}{1 \times 0.919} = +2.71°; [M]_D^{22} = +4.23° \text{ (homogeneous)}$$

3.465 mg. substance: 9.750 mg. CO_2 and 4.005 mg. H_2O
$C_{10}H_{20}O$. Calculated. C 76.8, H 12.8
156.16   Found.   " 76.7, " 12.9

Dextro-1-Bromo-3-Cyclohexylbutane—10 gm. of 3-cyclohexylbutanol-1, $[\alpha]_D^{22} = +2.71°$ (homogeneous), were cooled in ice and 15 gm. of phosphorus tribromide were slowly added. The product was heated 15 minutes on a steam bath, cooled, and then poured on ice. The halide was extracted with ether, dried with sodium sulfate, and then the ether was distilled. The residue was shaken several times with cold concentrated sulfuric acid, separated, washed with water, and extracted with ether. The ether was

dried with sodium sulfate, then fractionated. B. p. 126° at 15 mm. Yield 11 gm. $n_D^{25} = 1.4911$; D $\frac{22}{4} = 1.155$.

$$[\alpha]_D^{22} = \frac{+6.01°}{1 \times 1.155} = +5.20°; [M]_D^{22} = +11.39° \text{ (homogeneous)}$$

5.642 mg. substance: 11.325 mg. CO_2 and 4.475 mg. H_2O
$C_{10}H_{19}Br$. Calculated. C 54.8, H 8.7
219.15   Found.   " 54.7, " 8.9

Dextro-Methylethylcyclohexylmethane (by Catalytic Reduction)— To 13.4 gm. of methylethylphenylmethane, $[\alpha]_D^{24} = -6.82°$ (homogeneous), was added 1 gm. of Adam's platinic oxide. The hydrocarbon was reduced by shaking with hydrogen under a pressure of 30 pounds. When absorption of hydrogen ceased, 25 gm. of glacial acetic acid and 0.5 gm. of additional catalyst were added and the shaking continued several hours to insure complete reduction. The acetic acid solution was filtered and shaken with sodium carbonate solution. The hydrocarbon was extracted with ether and the ether solution dried with dry sodium sulfate. The ether was evaporated and the hydrocarbon distilled from sodium. B. p. 174° at 760 mm. Yield 9 gm. D $\frac{22}{4} = 0.810$.

$$[\alpha]_D^{22} = \frac{+0.64°}{1 \times 1.810} = +0.79°; [M]_D^{22} = +1.11° \text{ (homogeneous)}$$

3.535 mg. substance: 11.105 mg. CO_2 and 4.595 mg. H_2O
$C_{10}H_{20}$. Calculated. C 85.6, H 14.3
140.16   Found.   " 85.7, " 14.5

*Resolution of 2-Cyclohexylbutyric Acid-4—*The inactive acid was prepared from methylcyclohexylbromomethane and malonic ester.

The acid was dissolved in boiling acetone containing 10 per cent water and an equivalent quantity of quinine was added. This was cooled to −15°. After four crystallizations the quinine salt was decomposed by dilute hydrochloric acid and the organic acid extracted with ether. It was then distilled. B. p. 145° at 4 mm. D $\frac{24}{4} = 1.018$.

$$[\alpha]_D^{24} = \frac{-0.76°}{1 \times 1.018} = -0.75°; [M]_D^{24} = -1.26° \text{ (homogeneous)}$$

3.865 mg. substance: 9.985 mg. CO_2 and 3.736 mg. H_2O
$C_{10}H_{18}O_2$. Calculated. C 70.5, H 10.6
170.14   Found.   " 70.5, " 10.8

Levo-Ethyl Ester of 2-Cyclohexylbutyric Acid-4—100 gm. of 2-cyclohexylbutyric acid-4, $[M]_D^{24} = -1.26°$ (homogeneous), were dissolved in 250 cc. of absolute alcohol and 5 cc. of concentrated sulfuric acid were added. The mixture was heated 30 minutes on a steam bath. The excess alcohol was distilled off, water was added, and the ester extracted with ether. This was shaken with sodium carbonate solution, then dried with sodium sulfate. The ether was evaporated and the ester distilled. B. p. 104° at 3 mm. Yield 104 gm. D $\frac{28}{4}$ = 0.937.

$$[\alpha]_D^{28} = \frac{-1.35°}{1 \times 0.937} = -1.44°; [M]_D^{28} = -2.88° \text{ (homogeneous)}$$

4.240 mg. substance: 11.324 mg. CO_2 and 4.325 mg. H_2O.
$C_{12}H_{22}O_2$.   Calculated.   C 72.7, H 11.1
198.18   Found.   " 72.8, " 11.4

Levo-2-Cyclohexylbutanol-4—104 gm. of ethyl ester of 2-cyclohexylbutyric acid-4, $[\alpha]_D^{28} = -1.44°$ (homogeneous), were dissolved in 350 cc. of absolute ethyl alcohol. This was slowly dropped into a suspension of 120 gm. of sodium in 700 cc. of boiling toluene with rapid stirring. After addition was complete, the excess sodium was destroyed by dilute alcohol, water was added, and the toluene solution separated and well washed. The carbinol was separated by distillation. B. p. 128° at 15 mm. Yield 70 gm. $D\frac{27}{4} = 0.915$.

$$[\alpha]_D^{27} = \frac{-2.46°}{1 \times 0.915} = -2.69°; [M]_D^{27} = -4.20° \text{ (homogeneous)}$$

3.551 mg. substance: 10.001 mg. CO_2 and 4.116 mg. H_2O.
$C_{10}H_{20}O$.   Calculated.   C 76.9, H 12.9
156.16   Found.   " 76.8, " 13.0

Levo-4-Bromo-2-Cyclohexylbutane—70 gm. of 2-cyclohexylbutanol-4, $[M]_D^{27} = -4.20°$ (homogeneous), were cooled in ice and 100 gm. of phosphorus tribromide were slowly added. The product was heated 30 minutes on a steam bath, cooled, and then poured on ice. The halide was extracted with ether; the ether was evaporated and the residue treated with cold concentrated

sulfuric acid as previously described. B. p. 135° at 15 mm.
Yield 72 gm.   D $\frac{27}{4}$ = 1.142.

$$[\alpha]_D^{27} = \frac{-7.53°}{1 \times 1.142} = -6.60°; [M]_D^{27} = -15.18° \text{ (homogeneous)}$$

3.958 mg. substance: 7.870 mg. CO_2 and 3.226 mg. H_2O
$C_{10}H_{19}Br$.   Calculated.   C 54.7, H 8.7
219.15   Found.   " 54.2, " 9.1

Dextro-2-Cyclohexylbutane—A Grignard reagent was prepared
from 2 gm. of magnesium in 75 cc. of dry ether and 15 gm. of
1-bromo-3-cyclohexylbutane, $[M]_D^{27} = +6.36°$ (homogeneous).
This was poured on ice and the hydrocarbon extracted with ether.
The ether was distilled and the residue shaken with cold concen-
trated sulfuric acid. It was washed with sodium carbonate solu-
tion, dried, and then distilled from a small piece of sodium. The
distillate was shaken with platinum under hydrogen to insure
complete reduction, then redistilled. B. p. 174° at 760 mm.
Yield 3.2 gm.   D $\frac{27}{4}$ = 0.805.

$$[\alpha]_D^{27} = \frac{+0.36°}{1 \times 0.805} = +0.45°; [M]_D^{27} = +0.63° \text{ (homogeneous)}$$

4.342 mg. substance: 13.616 mg. CO_2 and 5.568 mg. H_2O
$C_{10}H_{20}$.   Calculated.   C 85.6, H 14.3
140.16   Found.   " 85.5, " 14.3

Levo-2-Cyclohexylvaleric Acid-5—A Grignard reagent was
prepared from 11 gm. of magnesium in 250 cc. of dry ether and
70 gm. of 4-bromo-2-cyclohexylbutane, $[M]_D^{27} = -5.21°$ (homo-
geneous). The Grignard reagent was cooled in ice and an excess
of dry carbon dioxide was passed in. The solution was poured on
ice and hydrochloric acid and the organic acid extracted with
ether. The ether was distilled and the acid purified through its
sodium salt. B. p. 149° at 3 mm. Yield 51 gm.   D $\frac{27}{4}$ = 0.996.

$$[\alpha]_D^{27} = \frac{-2.16°}{1 \times 0.996} = -2.17°; [M]_D^{27} = -4.02° \text{ (homogeneous)}$$

3.519 mg. substance: 9.255 mg. CO_2 and 3.277 mg. H_2O
$C_{11}H_{20}O_2$.   Calculated.   C 71.7, H 10.9
184.16   Found.   " 71.7, " 10.4

Levo-Ethyl Ester of 2-Cyclohexylvaleric Acid-5—51 gm. of 2-cyclohexylvaleric acid-5, $[M]_D^{27} = -4.02°$ (homogeneous), were dissolved in 100 cc. of absolute ethyl alcohol and 5 cc. of concentrated sulfuric acid were added. The product was heated $\frac{1}{2}$ hour on a steam bath, the excess alcohol distilled off, and then water was added. The ester was extracted with ether. The ethereal solution was shaken with sodium carbonate solution, then dried with sodium sulfate, and distilled. B. p. 133° at 7 mm. Yield 52 gm. D $\frac{27}{4} = 0.928$.

$$[\alpha]_D^{27} = \frac{-1.98°}{1 \times 0.928} = -2.14°; [M]_D^{27} = -4.54° \text{ (homogeneous)}$$

3.392 mg. substance: 9.089 mg. CO_2 and 3.455 mg. H_2O
$C_{13}H_{24}O_2$.   Calculated.   C 73.5, H 11.3
212.2   Found.   " 73.0, " 11.4

Levo-2-Cyclohexylpentanol-5—51 gm. of the ethyl ester of 2-cyclohexylvaleric acid-5, $[M]_D^{27} = -4.54°$ (homogeneous), were dissolved in 200 cc. of absolute ethyl alcohol. This was slowly dropped into a suspension of 70 gm. of sodium in 350 cc. of boiling toluene with vigorous stirring. When reduction was complete, the product was poured into water and the toluene layer separated. This was washed with water, dried, then distilled. B. p. 134° at 15 mm. Yield 29 gm. D $\frac{27}{4} = 0.907$.

$$[\alpha]_D^{27} = \frac{-2.70°}{1 \times 0.907} = -2.98°; [M]_D^{27} = -5.04° \text{ (homogeneous)}$$

3.573 mg. substance: 10.100 mg. CO_2 and 4.278 mg. H_2O
$C_{11}H_{22}O$.   Calculated.   C 77.5, H 12.9
170.18   Found.   " 77.1, " 13.4

Levo-2-Cyclohexyl-5-Bromopentane—29 gm. of 2-cyclohexylpentanol-5, $[M]_D^{27} = -5.04°$ (homogeneous), were cooled in ice and 50 gm. of phosphorus tribromide were slowly added. The mixture was heated on a steam bath for 20 minutes, then cooled, and poured on ice. The halide was extracted with ether and then distilled without further purification. B. p. 146° at 16 mm. Yield 33 gm. D $\frac{27}{4} = 1.137$.

$$[\alpha]_D^{27} = \frac{-7.46°}{1 \times 1.137} = -6.56°; [M]_D^{27} = -14.86° \text{ (homogeneous)}$$

5.826 mg. substance: 12.228 mg. CO_2 and 4.747 mg. H_2O
$C_{11}H_{21}Br$.   Calculated.   C 56.5, H 9.1
233.17   Found.   " 57.2, " 9.1

Levo-2-Cyclohexylpentane—33 gm. of 2-cyclohexyl-5-bromo-pentane, $[M]_D^{27} = -14.86°$ (homogeneous), were dropped into 3 gm. of magnesium in 100 cc. of dry ether. When the Grignard reaction was complete, the reaction product was decomposed by pouring on ice and hydrochloric acid. The hydrocarbon was extracted, then purified as described for 2-cyclohexylbutane. B. p. 88° at 15 mm. Yield 5 gm. D $\frac{27}{4}$ = 0.814.

$$[\alpha]_D^{27} = \frac{-0.66°}{1 \times 0.814} = -0.81°; [M]_D^{27} = -1.25° \text{ (homogeneous)}$$

3.157 mg. substance: 9.911 mg. CO_2 and 4.120 mg. H_2O
$C_{11}H_{22}$.   Calculated.   C 85.6, H 14.3
154.18   Found.   " 85.6, " 14.6

Levo-2-Cyclohexylhexane (by Catalytic Reduction)—18 gm. of 2-phenylhexane, $[\alpha]_D^{25} = +1.96°$ (homogeneous), were dissolved in 25 cc. of glacial acetic acid and 1 gm. of platinum oxide was added. The hydrocarbon was reduced by shaking with hydrogen under 30 pounds pressure until absorption ceased. Fresh catalyst was added and the shaking continued 3 hours longer. The material was filtered, shaken with sodium carbonate solution, extracted with ether, and the ether distilled. The residue was distilled from sodium after purification as described for 2-cyclohexylbutane. B. p. 101° at 18 mm. Yield 15 gm. D $\frac{25}{4}$ = 0.823.

$$[\alpha]_D^{25} = \frac{-0.74°}{1 \times 0.823} = -0.90°; [M]_D^{25} = -1.51° \text{ (homogeneous)}$$

4.174 mg. substance: 13.123 mg. CO_2 and 5.405 mg. H_2O
$C_{12}H_{24}$.   Calculated.   C 85.6, H 14.3
168.2   Found.   " 85.7, " 14.5

Levo-3-Cyclohexylvaleric Acid-5 (by Catalytic Reduction)—10 gm. of 3-phenylvaleric acid-5, $[\alpha]_D^{27} = \frac{-3.50° \times 100}{1.23 \times 1 \times 20} = -14.2°$

(in benzene), were dissolved in 15 cc. of glacial acetic acid.   0.5 gm. of platinum oxide was added and the acid reduced by shaking with hydrogen under a pressure of 30 pounds.   When absorption ceased, fresh catalyst was added and shaking continued but the mixture absorbed no more hydrogen.   Ether was added and the catalyst filtered from the solution.   It was then distilled.   B. p. 153° at 5 mm.   Yield 6 gm.   D $\frac{27}{4}$ = 0.996.   An ultra-violet absorption test on this showed that it contained no trace of un-reduced phenyl compound.

$$[\alpha]_D^{27} = \frac{-1.02}{1 \times 0.996} = -1.53°; [M]_D^{27} = -2.82° \text{ (homogeneous)}$$

(The above was repeated, giving the same results on reduction.)

3.678 mg. substance: 9.706 mg. CO_2 and 3.762 mg. H_2O
$C_{11}H_{20}O_2$.   Calculated.   C 71.7, H 11.0
184.16   Found.   " 71.9, " 11.4

Dextro-3-Cyclohexylpentanol-1 (by Catalytic Reduction)—16.4 gm. of 3-phenylpentanol-1, $[\alpha]_D^{25}$ = −4.54° (homogeneous), were dissolved in 25 cc. of glacial acetic acid and 1 gm. of platinum oxide was added.   Reduction was carried out as described for 3-cyclohexylbutanol-1.   B. p. 135° at 15 mm.   Yield 15 gm.   D $\frac{25}{4}$ = 0.911.

$$[\alpha]_D^{25} = \frac{+2.02°}{1 \times 0.911} = +2.22°; [M]_D^{25} = +3.78° \text{ (homogeneous)}$$

4.575 mg. substance: 12.980 mg. CO_2 and 5.245 mg. H_2O
$C_{11}H_{22}O$.   Calculated.   C 77.6, H 13.0
170.18   Found.   " 77.4, " 12.8

Dextro-1-Bromo-3-Cyclohexylpentane—10 gm. of 3-cyclohexyl-pentanol-1, $[\alpha]_D^{25}$ = +2.22° (homogeneous), were cooled in ice and 15 gm. of phosphorus tribromide slowly added.   The solution was then heated $\frac{1}{2}$ hour on a steam bath, poured on ice, and ex-tracted with ether.   After the usual purification the halide was distilled.   B. p. 135° at 15 mm.   Yield 10 gm.   D $\frac{25}{4}$ = 1.145.

$$[\alpha]_D^{25} = \frac{+3.16°}{1 \times 1.145} = +2.76°; [M]_D^{25} = +6.43° \text{ (homogeneous)}$$

4.840 mg. substance: 10.050 mg. CO_2 and 3.980 mg. H_2O
$C_{11}H_{21}Br$.   Calculated.   C 56.6, H 9.1
233.17   Found.   " 56.6, " 9.2

Dextro-Ethyl-n-Propylcyclohexylmethane (by Catalytic Reduction)
— 16 gm. of ethylpropylphenylmethane, $[M]_D^{21} = +0.92°$ (homogeneous), were dissolved in 25 cc. of glacial acetic acid and 1 gm. of platinum oxide was added. This was reduced by shaking with hydrogen under a pressure of 30 pounds. After absorption of hydrogen ceased, fresh catalyst was added and shaking continued several hours, but there was apparently no further absorption of hydrogen. The hydrocarbon was isolated and purified as described for 2-cyclohexylbutane. B. p. 111° at 28 mm. Yield 13 gm. $D \frac{23}{4} = 0.823$.

$$[\alpha]_D^{23} \quad \frac{-0.73°}{1 \times 0.823} = -0.89°; [M]_D^{23} = -1.50° \text{ (homogeneous)}$$

4.835 mg. substance: 15.215 mg. CO_2 and 6.240 mg. H_2O
$C_{12}H_{24}$. Calculated. C 85.6, H 14.4
168.2   Found.   " 85.7, " 14.4

Levo-3-Cyclohexylheptane (by Catalytic Reduction)—17 gm. of 3-phenylheptane, $[\alpha]_D^{25} = +0.97°$ (homogeneous), were dissolved in 25 gm. of glacial acetic acid and 1 gm. of platinic oxide was added. The hydrocarbon was reduced by shaking with hydrogen under a pressure of 30 pounds. Reduction was complete in 1 hour but was continued for 20 hours to insure complete reduction. The solution was filtered from platinum and the acetic acid shaken out with sodium carbonate solution. The hydrocarbon was extracted with ether, then distilled from sodium. B. p. 112° at 15 mm. Yield 15 gm. $D \frac{25}{4} = 0.819$.

$$[\alpha]_D^{25} = \frac{-0.56°}{1 \times 0.819} = -0.68°; [M]_D^{25} = -1.25° \text{ (homogeneous)}$$

3.233 mg. substance: 10.208 mg. CO_2 and 4.125 mg. H_2O
$C_{13}H_{26}$. Calculated. C 85.6, H 14.4
182.21   Found.   " 86.1, " 14.2

Resolution of 3-Cyclohexylvaleric Acid-5—The inactive acid was prepared from ethylcyclohexylbromomethane and ethyl malonate.

The inactive acid was resolved by recrystallizing its quinine

salt from acetone as described for 2-cyclohexylbutyric acid-4. B. p. 148° at 4 mm.   Yield 10 gm.   D $\frac{27}{4}$ = 0.996.

$$[\alpha]_D^{27} = \frac{-1.37°}{1 \times 0.996} = -1.38°; [M]_D^{27} = -2.54° \text{ (homogeneous)}$$

4.689 mg. substance: 12.355 mg. CO_2 and 4.520 mg. H_2O
$C_{11}H_{20}O_2$.   Calculated.   C 71.7, H 11.0
184.16   Found.   " 71.8, " 10.8

Levo-Ethyl Ester of 3-Cyclohexylvaleric Acid-5—10 gm. of 3-cyclohexylvaleric acid-5, $[\alpha]_D^{27}$ = −1.38° (homogeneous), were mixed with 20 cc. of absolute ethyl alcohol and 3 cc. of sulfuric acid, and the product was heated on a steam bath for 15 minutes. Some of the alcohol was distilled off and ice was added.   The ester was extracted with ether and shaken with sodium carbonate solution.   It was dried, then distilled.   B. p. 118° at 6 mm.   Yield 11 gm.   D $\frac{27}{4}$ = 0.931.

$$[\alpha]_D^{27} = \frac{-0.76°}{1 \times 0.931} = -0.82°; [M]_D^{27} = -1.74° \text{ (homogeneous)}$$

3.322 mg. substance: 9.002 mg. CO_2 and 3.391 mg. H_2O
$C_{13}H_{24}O_2$.   Calculated.   C 73.5, H 11.3
212.2   Found.   " 73.9, " 11.4

Dextro-3-Cyclohexylpentanol-1—10 gm. of ethyl ester of 3-cyclohexylvaleric acid-5, $[\alpha]_D^{27}$ = −0.82° (homogeneous), were dissolved in 25 cc. of absolute ethyl alcohol and this was dropped into a suspension of 15 gm. of sodium in 75 cc. of boiling toluene.   The product was isolated as described for 3-cyclohexylbutanol-1.   B. p. 119° at 5 mm.   Yield 5 gm.   D $\frac{27}{4}$ = 0.909.

$$[\alpha]_D^{27} = \frac{+1.82°}{1 \times 0.909} = +2.00°; [M]_D^{27} = +3.40° \text{ (homogeneous)}$$

3.830 mg. substance: 10.875 mg. CO_2 and 4.591 mg. H_2O
$C_{11}H_{22}O$.   Calculated.   C 77.6, H 13.0
170.18   Found.   " 77.4, " 13.4

Levo-4-Cyclohexylcaproic Acid-6 (by Catalytic Reduction)—20 gm. of 4-phenylcaproic acid-6, $[\alpha]_D^{24}$ = −2.48° (homogeneous), were dissolved in 50 gm. of glacial acetic acid and 1 gm. of plati-

num oxide was added. Reduction was carried out as described for 2-cyclohexylbutyric acid-4. B. p. 155° at 4 mm. Yield 15 gm. D $\frac{24}{4}$ = 0.982.

$$\frac{-0.77°}{2 \times 0.982} : -0.39°; [M]_D^{24} = -0.78° \text{ (homogeneous)}$$

4.600 mg. substance: 12.282 mg. CO_2 and 4.660 mg. H_2O
$C_{12}H_{22}O_2$. Calculated. C 72.7, H 11.2
198.17   Found.   " 72.8, " 11.3

Levo-3-Cyclohexylhexanol-1 (by Catalytic Reduction)—18 gm. of 3-phenylhexanol-1, $[\alpha]_D^{25}$ = +2.51° (homogeneous), were dissolved in 25 cc. of glacial acetic acid and 1 gm. of platinum oxide was added. This was shaken with hydrogen under 30 pounds pressure until absorption was complete. Fresh catalyst was then added and the product was shaken several hours longer to insure complete reduction. It was then isolated as described for 3-cyclohexylbutanol-1. B. p. 141° at 15 mm. Yield 17 gm. D $_4$

$$[\alpha]_D^{25} = \frac{-0.17°}{1 \times 0.916} = -0.19°; [M]_D^{25} = -0.34° \text{ (homogeneous)}$$

4.465 mg. substance: 12.810 mg. CO_2 and 5.140 mg. H_2O
$C_{12}H_{24}O$. Calculated. C 78.2, H 13.1
184.2   Found.   " 78.2, " 12.9

Levo-1-Bromo-3-Cyclohexylhexane—12 gm. of 3-cyclohexyl-hexanol-1, $[\alpha]_D^{25}$ = −0.19° (homogeneous), were cooled in ice and 20 gm. of phosphorus tribromide were added. Bromination was carried out as described for 1-bromo-3-cyclohexylbutane. B. p. 145° at 15 mm. Yield 12 gm. D $\frac{25}{4}$ = 1.083.

$$[\alpha]_D^{25} = \frac{-0.67°}{1 \times 1.083} = -0.62°; [M]_D^{25} = -1.53° \text{ (homogeneous)}$$

4.625 mg. substance: 9.910 mg. CO_2 and 3.845 mg. H_2O
$C_{12}H_{23}Br$. Calculated. C 58.3, H 9.4
247.18   Found.   " 58.4, " 9.3

4-Cyclohexyloctane (by Catalytic Reduction)—20 gm. of 4-phenyl-octane, $[M]_D^{25}$ = +1.18° (homogeneous), were dissolved in 25 cc.

of glacial acetic acid and reduced by shaking with 1 gm. of platinic oxide under hydrogen at a pressure of 30 pounds. When absorption of hydrogen ceased, fresh catalyst was added but there was no further absorption of hydrogen. The acetic acid was shaken out with sodium carbonate solution and the hydrocarbon extracted with ether, and then distilled from sodium. B. p. 123° at 15 mm. Yield 18 gm. D $\frac{27}{4}$ = 0.823.

$$[\alpha]_D^{25} = \frac{-0.24°}{1 \times 0.823} = -0.29°; [M]_D^{25} = -0.58° \text{ (homogeneous)}$$

3.506 mg. substance: 11.015 mg. CO_2 and 4.485 mg. H_2O
$C_{14}H_{28}$. Calculated. C 85.62, H 14.38
196.22 Found. " 85.67, " 14.31

Levo-4-Cyclohexylcaproic Acid-6—The inactive acid was prepared from *n*-propylcyclohexylbromomethane and ethyl malonate. This was dissolved in 3 volumes of boiling acetone and 1 equivalent of quinine was added. To this was added 20 per cent water. The solution was cooled at $-15°$ until crystallization was complete. The resolution was very difficult. B. p. 155° at 4 mm. Yield 65 gm. D $\frac{24}{4}$ = 0.982.

$$[\alpha]_D^{24} = \frac{+2.00°}{1 \times 0.982} = +2.04°; [M]_D^{24} = +4.04° \text{ (homogeneous)}$$

5.216 mg. substance: 14.019 mg. CO_2 and 5.109 mg. H_2O
$C_{12}H_{22}O_2$. Calculated. C 72.7, H 11.2
198.18 Found. " 73.2, " 11.0

Levo-Ethyl Ester of 4-Cyclohexylcaproic Acid-6—64 gm. of 4-cyclohexylcaproic acid-6, $[M]_D^{24} = +4.04°$ (homogeneous), were dissolved in 150 cc. of absolute alcohol and 5 cc. of concentrated sulfuric acid were added. The product was heated $\frac{1}{2}$ hour on a steam bath. The excess alcohol was distilled off and the ester isolated and purified in the usual way. B. p. 126° at 4 mm. Yield 61 gm. D $\frac{27}{4}$ = 0.934.

$$[\alpha]_D^{27} = \frac{+1.44°}{1 \times 0.934} = +1.54°; [M]_D^{27} = +3.90° \text{ (homogeneous)}$$

3.429 mg. substance: 9.337 mg. CO_2 and 3.671 mg. H_2O
$C_{14}H_{26}O_2$. Calculated. C 74.3, H 11.6
226.24 Found. " 74.3, " 11.9

Dextro-3-Cyclohexyl-1-Hexanol—60 gm. of ethyl ester of 4-cyclohexylcaproic acid-6, $[M]_D^{27} = +3.92°$ (homogeneous), were dissolved in 200 cc. of absolute alcohol and this slowly dropped into a suspension of 80 gm. of sodium in 400 cc. of toluene with rapid stirring. After reduction was complete, the carbinol was extracted, then distilled. B. p. 127° at 4 mm. Yield 35 gm. $D \frac{25}{4} = 0.916$.

$$[\alpha]_D^{25} = \frac{-0.72°}{1 \times 0.916} = -0.79°; [M]_D^{25} = -1.42° \text{ (homogeneous)}$$

3.109 mg. substance: 8.960 mg. CO_2 and 3.730 mg. H_2O

$C_{12}H_{24}O$.   Calculated.   C 78.2, H 13.1

184.2   Found.   " 78.6, " 13.4

Dextro-3-Cyclohexyl-1-Bromohexane—100 gm. of phosphorus tribromide were slowly added with cooling to 35 gm. of 3-cyclohexyl-1-hexanol, $[M]_D^{25} = -1.44°$ (homogeneous). The product was heated $\frac{1}{2}$ hour on a steam bath, cooled, and poured on ice. The halide was extracted with ether, then distilled. B. p. 145° at 15 mm. Yield 41 gm. $D \frac{25}{4} = 1.043$.

$$[\alpha]_D^{25} = \frac{-1.44°}{1 \times 1.043} = -1.38°; [M]_D^{25} = -3.40° \text{ (homogeneous)}$$

4.608 mg. substance: 9.885 mg. CO_2 and 3.925 mg. H_2O

$C_{12}H_{23}Br$.   Calculated.   C 58.3, H 9.4

247.19   Found.   " 58.5, " 9.5

Dextro-3-Cyclohexylhexane—A Grignard reagent was prepared from 40 gm. of 3-cyclohexyl-1-bromohexane, $[M]_D^{25} = -3.44°$ (homogeneous), and 4 gm. of magnesium in 100 cc. of dry ether. This was poured on ice and hydrochloric acid. The hydrocarbon was extracted and purified as previously described. It was then distilled from sodium. B. p. 111° at 28 mm. Yield 15 gm. $D \frac{23}{4} = 0.823$.

$$[\alpha]_D^{23} = \frac{+0.47°}{1 \times 0.823} = +0.57°; [M]_D^{23} = +0.96° \text{ (homogeneous)}$$

4.086 mg. substance: 12.890 mg. CO_2 and 5.150 mg. H_2O

$C_{12}H_{24}$.   Calculated.   C 85.6, H 14.4

168.2   Found.   " 86.0, " 14.1

Dextro-5-Cyclohexylheptanoic Acid-7—10 gm. of 5-phenyl-heptanoic acid-7, $[M]_D^{25} = +47.6°$ (homogeneous), were dissolved in 20 cc. of glacial acetic acid and 1 gm. of platinum oxide was added. This was shaken with hydrogen under a pressure of 25 pounds until absorption ceased. Fresh catalyst was added and shaking continued 8 hours longer. The product was filtered from the catalyst and then distilled. B. p. 155° at 2 mm.

$$[\alpha]_D^{25} = \frac{+4.40°}{1 \times 0.962} = +4.6°; [M]_D^{25} = +9.7° \text{ (homogeneous)}$$

4.741 mg. substance: 12.630 mg. CO_2 and 4.804 mg. H_2O
$\quad\quad\quad$ $C_{13}H_{24}O_2$. Calculated. C 73.5, H 11.4
$\quad\quad\quad$ 212.2 $\quad\quad$ Found. $\quad\quad$ " 72.6, " 11.3

Dextro-Methylethylphenylmethane—To 1 mol of methyl magnesium iodide in dry ether were added 60 gm. of ethylphenylchloromethane, $[M]_D^{24} = +30.62°$ (homogeneous). The Grignard reagent was allowed to stand overnight, then poured on ice and hydrochloric acid, and the hydrocarbon extracted with ether. This was purified as previously described by shaking with palladium and hydrogen at atmospheric pressure. B. p. 63° at 15 mm. Yield 6 gm. D $\frac{24}{4} = 0.868$.

$$[\alpha]_D^{24} = \frac{+1.02°}{1 \times 0.868} = +1.18°; [M]_D^{24} = +1.58° \text{ (homogeneous)}$$

3.065 mg. substance: 10.080 mg. CO_2 and 2.845 mg. H_2O
$\quad\quad\quad$ $C_{10}H_{14}$. Calculated. C 89.5, H 10.5
$\quad\quad\quad$ 134.11 $\quad\quad$ Found. $\quad\quad$ " 89.7, " 10.4

Dextro-5-Phenylheptanoic Acid-7—5 gm. of sodium were dissolved in 60 cc. of absolute alcohol and 30 gm. of ethyl malonate were added. This was followed by 35 gm. of *n*-butylphenylchloromethane, $[M]_D^{25} = -45.06°$ (homogeneous). The acid was isolated and purified as described for 2-phenylbutyric acid-4. B. p. 165° at 4 mm. Yield 10 gm.

$$[\alpha]_D^{25} = \frac{+7.99°}{1 \times 0.996} = +8.00°; [M]_D^{25} = +16.5° \text{ (homogeneous)}$$

4.731 mg. substance: 13.120 mg. CO_2 and 3.742 mg. H_2O
$\quad\quad\quad$ $C_{13}H_{18}O_2$. Calculated. C 75.7, H 8.8
$\quad\quad\quad$ 206.16 $\quad\quad$ Found. $\quad\quad$ " 75.6, " 8.8

Levo-Ethyl Ester of n-Butylphenylpropionic Acid—To 50 gm. of butylphenylpropionic acid, $[M]_D^{25} = -10.26°$ (homogeneous), were added 250 cc. of absolute alcohol and 5 cc. of concentrated sulfuric acid. The product was refluxed on a steam bath for $\frac{1}{2}$ hour, the excess alcohol distilled off, and then poured in water. The ester was extracted with ether, washed with sodium carbonate solution, then distilled. B. p. 128° at 2 mm. Yield 52 gm. D $\frac{25}{4}$ = 0.960.

$$[\alpha]_D^{25} = \frac{-2.86°}{1 \times 0.960} = -2.98°; [M]_D^{25} = -6.96° \text{ (homogeneous)}$$

3.705 mg. substance: 10.395 mg. CO_2 and 3.170 mg. H_2O
$$C_{15}H_{22}O_2. \quad \text{Calculated.} \quad \text{C } 76.8, \text{ H } 9.5$$
$$234.18 \qquad \text{Found.} \qquad \text{`` } 76.5, \text{`` } 9.6$$

Levo-3-Phenyl-1-Heptanol—50 gm. of ethyl ester of *n*-butyl-phenylpropionic acid, $[M]_D^{25} = -6.96°$ (homogeneous), were dissolved in 200 cc. of absolute alcohol and reduced by slowly dropping into a suspension of 70 gm. of sodium in 350 cc. of toluene with stirring. The carbinol was isolated and purified through the phthalic ester as previously described. B. p. 150° at 10 mm. Yield 34 gm. D $\frac{27}{4}$ = 0.947.

$$[\alpha]_D^{27} = \frac{-1.38}{1 \times 0.947} = -1.45°; [M]_D^{27} = -2.60° \text{ (homogeneous)}$$

3.710 mg. substance: 11.087 mg. CO_2 and 3.440 mg. H_2O
$$C_{13}H_{20}O. \quad \text{Calculated.} \quad \text{C } 81.2, \text{ H } 10.5$$
$$192.16 \qquad \text{Found.} \qquad \text{`` } 81.5, \text{`` } 10.4$$

Levo-3-Phenyl-1-Bromoheptane—33 gm. of 3-phenyl-1-heptanol, $[M]_D^{27} = -2.64°$ (homogeneous), were cooled in ice and 50 gm. of phosphorus tribromide added. The halide was prepared and purified in the usual way. B. p. 132° at 4 mm. Yield 30 gm. D $\frac{27}{4}$ = 1.195.

$$[\alpha]_D^{27} = \frac{-12.42°}{1 \times 1.195} = -10.4°; [M]_D^{27} = -26.5° \text{ (homogeneous)}$$

3.989 mg. substance: 8.935 mg. CO_2 and 2.742 mg. H_2O
$$C_{13}H_{19}Br. \quad \text{Calculated.} \quad \text{C } 61.2, \text{ H } 7.5$$
$$255.15 \qquad \text{Found.} \qquad \text{`` } 61.1, \text{`` } 7.7$$

Dextro-3-Phenylheptane—A Grignard reagent was prepared from 30 gm. of 3-phenyl-1-bromoheptane, $[M]_D^{27} = -20.4°$ (homogeneous), and 3 gm. of magnesium in ether. The ethereal solution was poured on ice and hydrochloric acid. The crude hydrocarbon was distilled, then purified by shaking with colloidal palladium and hydrogen. B. p. 112° at 15 mm. Yield 10 gm. $D\frac{25}{4} = -0.856$.

$$[\alpha]_D^{25} = \frac{+0.64°}{1 \times 0.856} = +0.75°; [M]_D^{25} = +1.32° \text{ (homogeneous)}$$

3.885 mg. substance: 12.589 mg. CO_2 and 3.946 mg. H_2O
$C_{13}H_{20}$.   Calculated.   C 88.5, H 11.4
176.16   Found.   " 88.4, " 11.4

Levo-3-Cyclohexylheptane—8 gm. of 3-phenylheptane, $[M]_D^{25} = +1.32°$ (homogeneous), were dissolved in 25 cc. of glacial acetic acid and 1 gm. of platinic oxide was added. The product was shaken under an atmosphere of hydrogen at 30 pounds pressure until reduction was complete. The hydrocarbon was isolated in the usual way. Yield 6 gm. B. p. 112° at 15 mm. $D\frac{25}{4} = 0.819$.

$$[\alpha]_D^{25} = \frac{-0.44°}{1 \times 0.819} = -0.54°; [M]_D^{25} = -0.98° \text{ (homogeneous)}$$

3.601 mg. substance: 11.290 mg. CO_2 and 4.650 mg. H_2O
$C_{13}H_{26}$.   Calculated.   C 85.6, H 14.4
182.21   Found.   " 85.5, " 14.4

STUDIES IN GASTRIC SECRETION

IV. VARIATIONS IN THE CHLORINE CONTENT OF GASTRIC JUICE AND THEIR SIGNIFICANCE*

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INTRODUCTION

The present investigation is a study of the variations in the concentrations of both total and neutral chloride of gastric juice, obtained from Pavlov pouch dogs by stimulation with either food or histamine. It differs from all such studies previously reported in that an attempt is here made to correlate these concentrations with the acidity of the secretion, instead of with its rate of flow or with the time interval between stimulation and collection of the secretion. This problem of the chlorine content of gastric juice derives its importance from two major sources: first and foremost, its bearing on the question of the source of the neutral chloride occurring in gastric juice; and second, its significance for an understanding of the mechanism of formation of hydrochloric acid. Because of its importance in these respects and also because of the failure of previous investigators to establish any significant regularity in connection with the problem, this reexamination of the nature and causes of variations in the concentration of gastric juice chlorides was undertaken.

A survey of the work published by numerous other investigators

of this subject fails to reveal any unanimity of opinion regarding it. Some of the workers with pure gastric juice have maintained that, even in the presence of wide variations in total acidity, the total concentration of chloride remains constant (Rosemann, 1907, 1911; Frouin and Gérard, 1912; Cohen, 1920; MacLean, Griffiths, and Williams, 1928; Bulger, Allen, and Harrison, 1928). In some of these papers the variation is small, whereas in others it is considerable; e.g., Cohen reports twenty-three chlorine values lying between 0.39 per cent and 0.54 per cent—a range equivalent to more than 30 per cent of the mean value. In contrast with these observers, others (Delhougne, 1926; Bliss, 1930) have recognized the existence of major variations in the chloride concentration, but these were always less than the corresponding variations in acidity. That these relatively small fluctuations may not be accidental but have a real significance is suggested by the further observations of Bliss that, following gastric stimulation the total chloride concentration rises to a maximum and then decreases, and that the curves for chloride and acid secretion are at least qualitatively parallel.

A similar parallelism of chloride and acidity values is observable in the results of numerous clinical investigators, following stimulation either by mechanical means or by test meal (Girardi and Cipriani, 1925; Polland, Roberts, and Bloomfield, 1928; Steinitz, 1928; Bliss, 1930). Another group of workers with human subjects (Pfaundler, 1900; Katsch and Kalk, 1926; Mirkin, Mogilewsky, and Rabinowitsch, 1927; Gorham, Stroud, and Huffman, 1928) have noted the occurrence of unmistakable variations in chloride content which, however, bore no relation to fluctuations in acidity. Most of these reports indicate that such irregular variations occur within far narrower limits than obtain for the corresponding acidity values. Finally, there have been a number of publications (MacLean and Griffiths, 1928; Bulger, Stroud, and Heideman, 1928; Rudd, 1930) in which it is maintained that the total chlorine concentration of stomach contents is approximately constant, even though the acidity manifest the usual changes.

Regarding variations in the concentration of neutral chloride (or fixed base, since anions other than chloride are virtually absent from gastric juice), several different views are current at present. Numerous investigators have shown that there exists a reciprocal

relation between the concentration of neutral chloride and the total acidity, whereby the one increases as the other decreases (Pfaundler, 1900; MacLean, Griffiths, and Williams, 1928; Bulger, Stroud, and Heideman, 1928; Close, 1929). More recently, it has been suggested that the neutral chloride-time curve is parallel to the corresponding pepsin activity-time curve (MacLean, Griffiths, and Hughes, 1929; Gilman and Cowgill, 1931). As yet, however, there has been no evidence to indicate the significance of these findings, or that either of these relations is a quantitative one.

In the present contribution it will be shown that for pure gastric juice (*i.e.* uncontaminated by food, saliva, regurgitated duodenal contents, and particularly blood and exudates arising from trauma) there exists a very simple mathematical relation between both the total and neutral chloride concentrations on one hand and the total acidity on the other. These relations are such as to be entirely in agreement with the views advanced in previous papers of this series concerning the nature of the parietal secretion and the process by which the usual extensive variations in stomach pouch acidity are brought about. Thus, the evidence will be shown to support a view regarding the several sources of the chloride radical in gastric juice which is part of a consistent picture of some of the processes involved in gastric secretion.

Methods

The following observations were made on five dogs, provided with fundic pouches of the Pavlov type. Some of the data were gotten by means of the *continuous collection* technique generally employed in such work; others were obtained by the *discontinuous collection* method, which depends on the retention of the gastric juice by a sphincter at the mouth of the pouch. The details of both these procedures, as well as the preparation and care of the animals, have already been described (Hollander and Cowgill, 1931). In conformity with the usual practice in this laboratory, every possible precaution was taken to prevent admixture with the gastric juice of blood or exudate, particularly any that might arise from abrasion of the mucosa.[1] The great importance of

[1] As a measure of the extent to which this high standard of technique was attained, it may be mentioned here that several samples of mucus-free juice

extreme caution in this respect cannot be emphasized too strongly if results possessing any reliable quantitative significance are to be obtained. In some of the experiments, food was employed as stimulus to secretion; in others, a subcutaneous injection of histamine. When the *discontinuous collection* procedure was used, the specific precautions designed to yield "constant acidity juice" were omitted, since it was desired to obtain samples, the acidities of which extend throughout the normal range ordinarily encountered.

Total acidity determinations were carried out by the micro titration method previously described (Hollander, 1931, a). Total chlorine determinations were performed by the method of Wilson and Ball (1928). Neutral chloride was calculated as the difference between the millimolar concentrations of total chloride and hydrochloric acid. Since the acid and chloride determinations are reliable to no better than 0.5 per cent, and the latter may even be as poor as 1.0 per cent, the reliability of the neutral chloride concentration calculated from these is probably no better than 1.5 per cent.

Observations

Total Chloride Concentration

In the first series of experiments, an attempt was made merely to repeat the observations of other investigators under carefully controlled conditions. Nineteen experiments were performed, the *continuous collection* technique and all five of the dogs being used. Both types of stimulus were employed to induce secretion. Samples were collected at convenient time intervals and acidity and total chloride determinations were made on all of them. The results, in millimolar concentration, were plotted against the times corresponding to the mid-points of the collection intervals. Twelve of these experiments manifested very good evidence for a qualitative parallelism of the acidity and chloride curves, irrespective of the conditions of the individual experiment. A very good example of this relation is shown in Fig. 1 (Experiment B-57),

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obtained from one of these dogs showed a complete absence of phosphate when tested by the method of Fiske and Subbarow. The further significance of this observation will be discussed in the next paper of this series.

in which it can be seen that, with but one exception (associated with a period of vomiting), every rise or fall in acidity is accompanied by a like change in chloride concentration. In all the experiments, however, the total range of variation for chloride was very much less than that for acidity.

In the seven experiments in which this parallel relation between acid and chloride concentrations was not so apparent, masking of the parallelism may be accounted for in the following way. The experimental errors in the analytical methods, as we have already indicated, are relatively large; in the case of the neutral chloride values, this error may be 2 millimols or more. Also, in addition
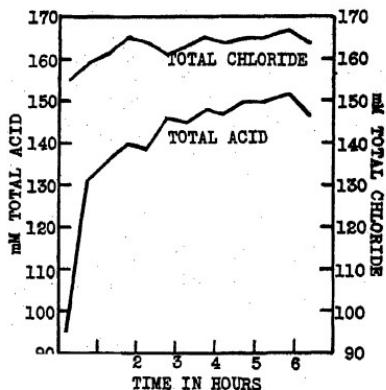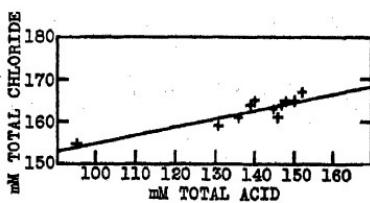
FIG. 1. Total chloride and acidity as functions of time; Experiment B-57.
FIG. 2. Total chloride as a function of acidity; Experiment B-57.

to the accidental variations which are notoriously inherent in all work with living animals, there are numerous specific uncontrollable factors which obtain in the present work. Among these are the variations in the proportions of mucus, enzyme secretions, and other constituents which may be mixed with the pure parietal secretion; also, changes occur in the osmotic pressure of the blood, arising from the various processes which accompany digestion and secretion, and which exert a wholly unpredictable influence on the total electrolyte concentration of gastric juice throughout any one experiment. Consequently, we are dealing here with a succession of chloride values which differ among themselves by only small amounts, and upon which there is superimposed an aggregate

of accidental errors of considerable magnitude relative to the range of these chloride values. In the case of the seven experiments which manifested no parallelism, this range was particularly small, and therefore the influence of such random variations might easily be sufficient to change the directions (up or down) of some of the line segments joining the successive chloride points in the graphs. Such a shift in the directions of only a few of the line segments would serve to disrupt entirely the parallelism which is so obvious in a majority of the experiments. It may be concluded, therefore, that our results verify the observations of previous investigators who have reported a qualitative parallelism between the acid and chloride concentrations in gastric juice. At the same time, the large experimental errors serve to explain why so many other workers have reported either variations in chloride values uncorrelated with acidity, or else an ostensible constancy of this concentration factor.

Such a parallelism suggests the existence of an exact quantitative relation between chloride and acid concentrations, although such experiments have never been analyzed and studied from this point of view. Therefore, the same data were replotted with the *exclusion of time as a variable; i.e.,* total chloride was plotted directly against total acidity. The result, in each case, was a straight line within reasonably narrow limits. In the illustration in Fig. 2, plotted from the same data as Fig. 1, no point deviates from the line by more than 3 millimols. Further, these deviations are entirely random; *i.e.,* they manifest no systematic trend away from the straight line.

Now, the failure of the individual points to show an even closer approximation to the straight line than they do may be ascribed to these same factors which were discussed above as ordinarily contributing to the accidental errors in these experiments. In order to reduce the influence of these accidental and variable factors, the logical procedure would be to combine the results of a large number of such experiments and subject them to statistical treatment. This method, however, demands such a large body of data that it is made prohibitive by the practical difficulties involved in carrying out so many experiments. Accordingly, a highly simplified procedure was employed instead. The chlorine and acid values for the 121 points of all nineteen experiments were

first arranged in the order of increasing acidity, and independently of the conditions of the original experiments. They were then grouped in such a way that all the chlorine values corresponding to acidities 100 to 109 mM were placed in the first group, acidities 110 to 119 mM in the second, and so on to the seventh group of acidities 160 to 169 mM. When the averages for both acid and chloride values within each group were calculated and the results were plotted (Fig. 3), they yielded a straight line from which no point deviated by more than about 1.5 mM. This result is all the more striking because it embodies the data of those experiments which showed no parallelism in the first place. It follows, therefore, that an analysis of the combined data of all nineteen experiments by this method of averages confirms the conclusion to be drawn from a majority of them when studied individually.
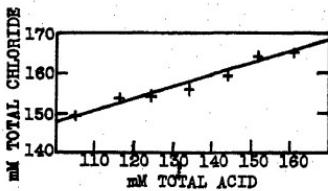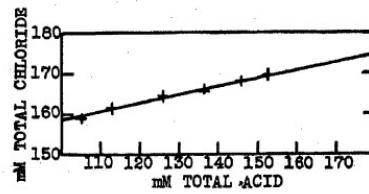
FIG. 3. Total chloride as a function of acidity; mean curve for nineteen continuous collection experiments.

FIG. 4. Total chloride as a function of acidity; mean curve for Experiment C-19, a discontinuous collection experiment.

Since the first series, consisting entirely of experiments by the *continuous collection* technique, gave such striking results, it was next attempted to make a similar analysis of data obtained by the *discontinuous collection* procedure. Accordingly, in the second series of experiments, three sets of such data were obtained from as many dogs, with various foods as stimuli. In some instances, the samples had been retained in the pouches for 12 or more hours. The data so obtained were then grouped and averaged as before, observations for each animal being treated independently of the others. The resulting graph from one set of such observations, containing a total of twenty-nine samples from Dog R, is shown in Fig. 4. In this particular case, the maximum deviation is only about 0.5 mM. It follows, therefore, that the straight line

relation between chloride and acid concentrations holds equally well for both methods of collecting gastric juice.

In final corroboration of the existence of this simple mathematical relation between the two variables, the same method of analysis with exclusion of the time factor was applied to some data from the work of another investigator. For this purpose a paper was chosen in which it was claimed that the chloride concentration of pouch juice is *constant* throughout any one experiment. The values from two single experiments on different dogs, reported by MacLean, Griffiths, and Williams (1928) in their Figs. 1 and 2, were used for this purpose. From the resulting graph (Fig. 5), there can be no doubt but that these observations also support
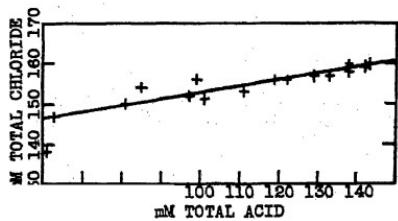
FIG. 5.   FIG. 6

FIG. 5. Total chloride as a function of acidity; data from two experiments by MacLean, Griffiths, and Williams.

FIG. 6. Neutral chloride as a function of acidity; Experiment B-57.
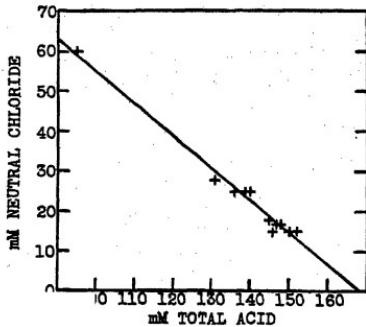
the conclusion established by means of our own data. Thus, additional evidence to support the existence of this quantitative relation can be adduced even from the work of other investigators who failed to seek it.

Neutral Chloride Concentration

The evidence so far presented relates only to the total concentration of chlorine in gastric juice. Possibly even more importance, however, attaches to the neutral chloride content; that is, all inorganic chlorides other than hydrochloric acid. It is generally assumed that organic chlorine is virtually absent from the gastric secretion; therefore, the difference between the concentrations of

total chloride and acid is usually taken as equal to the neutral
chloride concentration. Since no serious objection to this assump-
tion has ever been advanced, the same procedure has been em-
ployed in the present work. In each of Figs. 6, 7, and 8 there will
be found a graph of the total acidity plotted against the concentra-
tion of neutral chloride, calculated from the data of the correspond-
ing figures (Figs. 2 to 4, respectively). As might be anticipated
from the nature of the total chloride plots, the data in all three
cases fit straight lines extremely well. Although it has been
pointed out repeatedly by other investigators that the neutral
chloride concentration increases as the acidity decreases, it has
never before been shown that the relation is so precise a one as
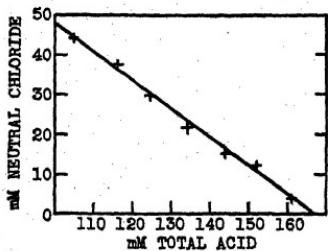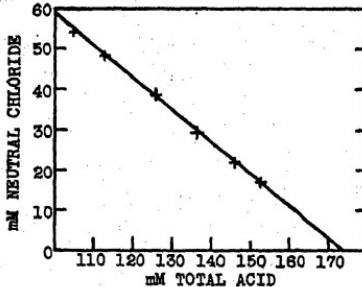
FIG. 7.   FIG. 8

FIG. 7. Neutral chloride as a function of acidity; mean curve for nineteen
continuous collection experiments.

FIG. 8. Neutral chloride as a function of acidity; mean curve for Experi-
ment C-19, a discontinuous collection experiment.

this. Thus it may be concluded that the neutral chloride con-
centration, as well as the total chloride concentration, is linked to
the acidity according to a straight line relation.

It is probable that the physiological connection between these
three variables is correspondingly simple, and that some indication
of its nature can be discovered from the characteristics of these
mathematical relations. Chief among these characteristics are
the following: (1) For both total and neutral chloride concentra-
tions, the graphs are straight lines. (2) These lines are not hori-
zontal but make angles with the acidity axis which are fairly
uniform for all the cases examined. These angles are given in
Columns 3 and 5 of Table I, under the headings α_{Cl} and α_{BCl}. The

measured values for the tangents of these angles, which represent the slopes of the lines, are given by m_{Cl} and m_{BCl} in Columns 4 and 6. (3) In Column 7 under the heading I_A, there are listed the intercepts of the neutral chloride lines with the acidity axis. For all the diverse cases here examined, these acidity values vary from 163 to 173 mM, which involves an extreme variation of only ± 3 per cent from the mean value of 168.

TABLE I

Characteristics of Straight Line Relations between Total Chloride, Neutral Chloride, and Total Acidity

α_{Cl} = the angle which the total chloride graph makes with the horizontal axis; α_{BCl} = the angle which the neutral chloride graph makes with the horizontal axis; m_{Cl} = tan α_{Cl}; m_{BCl} = tan α_{BCl}; I_A = the intercept of the neutral chloride graph with the acidity axis.

Experiment (1)	Fig. No. (2)	α_{Cl} (3)	m_{Cl} (4)	α_{BCl} (5)	m_{BCl} (6)	I_A (7)
		degrees		*degrees*		*mM*
B-57	2,6	10.9	0.19	128.9	−0.81	168
(19 experiments)	3,7	15.8	0.28	125.5	−0.72	167
C-19	4,8	11.2	0.20	128.8	−0.80	173
MacLean	5	9.2	0.16	130.0	−0.84	163
C-20	*	10.8	0.19	129.0	−0.81	173
C-25	*	18.4	0.33	123.8	−0.67	166

* These are the two discontinuous collection experiments, the graphs for which have not been presented here.

DISCUSSION

The chief interest in experiments such as those with which we are dealing here arises from the light which they may throw on the origin of the chloride radical in pure gastric juice. It is generally recognized that one major source of this element is the hydrochloric acid of the parietal secretion. It is only about the remaining portion, the so called neutral chloride, that there has been any extensive controversy.

On this question, investigators are divided into two schools. One of these groups maintains that the non-acid fraction also arises primarily from the parietal cells. The mechanism of acid secretion postulated by these workers involves the initial formation

of an isotonic solution of neutral chlorides from the blood, via the lymph and the parietal cytoplasm. These salts are then converted in varying degree to hydrochloric acid, the cation so liberated from the salt being reabsorbed and ultimately returned to the blood stream. Thus, the parietal secretion which enters the lumen of the stomach is an isotonic mixture of hydrochloric acid and neutral chlorides, in which the proportion of the latter decreases as the rate of secretion and the acidity both increase. This conception was first introduced by Rosemann (1907); since that time it has received support from an ever increasing number of investigators of gastric juice chlorides (Delhougne, 1926; Close, 1929; Duthrie, 1930).

In opposition to this school of opinion we have another which maintains that the neutral chloride of the gastric juice is derived from cells in the gastric mucosa other than the parietal cells. Beginning with Heidenhain and Pavlov, we find the view advanced that the parietal secretion contains a constant concentration of hydrochloric acid, and that all of the commonly observed fluctuations in acidity can be traced to the partial neutralization of this fluid by the mucous film which covers the mucosa at the beginning of secretion. In harmony with this conception, it has been maintained by many (among them Katsch and Kalk, 1926; Gamble and McIver, 1928; Bulger, Stroud, and Heideman, 1928; and McCann, 1929) that the neutral chlorides of the pure gastric juice are derived primarily from the mucous secretion rather than from the parietal secretion.

Recently, the Heidenhain-Pavlov theory was directly confirmed by Hollander and Cowgill (1931) who found that when gastric juice is collected from stomach pouch dogs in such a way as to reduce the mucous secretion to a minimum, the acidity actually is constant. The magnitude of this normal constant acidity was independent of the velocity of secretion, type of stimulus, individual animal, etc. In fact, evidence of various kinds, including the observations of Gamble and McIver (1928), suggested that it was determined predominantly by the osmotic concentration of the blood at the time of formation of the secretion. In contradistinction to the original theory, however, these observations indicated that the agent which causes the ordinary fluctuations in acidity of the pouch juice is elaborated continuously during

the secretory process, instead of being formed entirely before the beginning of hydrochloric acid secretion, as Pavlov believed. This agent consists of mucous secretion, peptic secretion, epithelial detritus, and possibly a lymph transudate, with the mucus playing by far the most important part.

In order to compare this modification of the Heidenhain-Pavlov theory with the results of the present investigation, it is necessary to extend the former still further so that it can be formulated quantitatively. Accordingly, as a preliminary working hypothesis, we conceive of gastric pouch juice as being a mixture of (1) the parietal secretion—which is an isotonic solution of hydrochloric acid containing no base and therefore no neutral chloride; and (2) a neutral or alkaline fluid—which is predominantly mucus. In the absence of data on the composition of pure gastric mucus, this fluid is assumed to be a colloidal dispersion of mucin in an isotonic mixture of neutral chlorides and bicarbonates, the concentration of the latter being smaller than that of the former. Other lymph constituents, like the phosphate radical, are also present, though in very small amounts.

When two such liquids are mixed, the acidity of the parietal secretion will be reduced by neutralization, with the loss of carbon dioxide, and also by dilution. The extent of this reduction will vary with the proportions of the components. Thus, the concentration of hydrochloric acid in the mixed gastric juice can possess any value between a maximum, corresponding to pure parietal secretion, and an "acid deficit", corresponding to the bicarbonate content of the mucous mixture. The total chloride concentration, however, will fluctuate within much narrower limits; the minimum value, fixed by the chloride concentration of the mucus, is not very much less than the maximum value, determined by the pure parietal secretion. The extent of this chloride range is equal to the bicarbonate concentration of the alkaline constituent. Since this is assumed to be less than half the total concentration of base in mucous, it follows that the decrease in total chloride suffered by the parietal fluid on admixture with the other will always be much less than the corresponding diminution in its total acidity value. As for the concentration of base in the mixed gastric juice, its value will fluctuate between zero and a maximum value corresponding to the unacidified mucous mixture.

Let us now examine some of the results of the present investigation, in the light of the opposing hypotheses advanced by Rosemann and by ourselves.

It has been found that, within the limits of error permitted by experimentation of this kind, the relation between total chloride concentration and acidity can be expressed by a straight line which is not parallel to the acidity axis. The occurrence of a straight line relation implies that the several factors which determine these concentrations are quantitative and exact; that is, variations in these concentrations are probably the result of a simple chemical or physiological process. That the line is not parallel with the acidity axis is especially significant, for if it were, it would betoken a constancy of total chloride concentration simultaneous with changes in acidity, such as the Rosemann hypothesis predicates. Fluctuations in the general osmotic equilibrium of the animal, according to this view, might manifest themselves by an increase in the random deviations of the individual points from the line, or by a vertical displacement of the line in its entirety; the general trend, however, would always conform to a horizontal line. Our hypothesis, on the contrary, implies that a decrease in acidity is always accompanied by a corresponding decrease in the total chloride concentration. The magnitude of this chloride change must invariably be less than that for the acidity. With these latter implications, the present observations conform completely.[2]

A second point of agreement between our present view of the secretion process and the facts can be demonstrated in the following way: according to our hypothesis, the maximum acidity attainable in gastric juice is that which corresponds to the pure parietal secretion, undiluted and unneutralized; this fluid is characterized by (1) the absence of fixed base, and (2) a concentration of hydrochloric acid which is isotonic with the blood. On the other hand, the intercept of the experimental neutral chloride graph with the acidity axis, which corresponds to a secreted fluid of zero salt

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[2] It may be of value here to point out that it has already been shown from acidity data alone (Hollander, 1931, b) that "the major argument on which Rosemann based his opposition to the Heidenhain-Pavlov theory" is fallacious. The present evidence only serves to complete the refutation of his picture of the secretory process.

concentration and the maximum attainable acidity, has a mean value of 167 mM for all the data of this study. But this acidity-intercept value is in good agreement with that concentration of hydrochloric acid which is isotonic with mammalian blood (hereafter designated as the *isosmotic concentration* of the acid).

Hill (1931) has estimated that the mean osmotic pressure of mammalian blood, calculated from data of Abderhalden on twelve different species, is equivalent to that of 0.1613 molal NaCl. This value is almost identical with the mean value for human blood as determined by Margaria (1930) under resting conditions. The freezing point depression corresponding to this concentration of NaCl is the same as that of a 157 mM HCl solution, as calculated from the data in the International Critical Tables. However, Margaria's values, regardless of sex, fluctuate within ±3 per cent of their mean; *i.e.*, 0.907 to 0.962 gm. of NaCl per 100 gm. of water. Further, under conditions of exercise, the freezing point depression may be increased by as much as 11 per cent. Since the dogs used in the present work were never maintained in a state of complete rest, it follows that a concentration of hydrochloric acid which exceeds this isosmotic value by only 6 per cent (*i.e.* 167 mM as compared with 157 mM) is unquestionably isotonic within the limits of experimental variation. Therefore, a sample of gastric juice containing no neutral chloride would be isotonic with mammalian blood, and no acidity significantly higher than the acid concentration of such a solution can be attained under normal conditions. Thus, by extrapolation from the observed data, it can be shown that the two most significant properties of the parietal secretion conform to the demands of our hypothesis.

Finally, a third point of agreement between the results of experiment and of theory can be established as follows: On the basis of a process of dilution and neutralization such as we have postulated, it is possible to develop equations for both total and neutral chlorides as functions of the acidity. Likewise, it is possible to set up equations which describe the experimental graphs for these variables. When this was done, it was found that the empirical and theoretical equations possess certain specific characteristics in common, thus establishing another point of accord between observation and hypothesis. The mathematical development follows.

Throughout the discussion, symbols with single primes refer to the parietal secretion, those with double primes to the mucus-containing mixture, and letters without any primes relate to the mixed gastric juice. The following symbols are used:

F. Hollander

C_{HCl} = total acidity
C_{Cl} = "   concentration of chloride
C_B = "   "   " base
C_{BCl} = concentration of neutral chloride
C_{BHCO_3} = "   " bicarbonate
C_o = the isosmotic concentration[3]

According to our theory, the parietal secretion is an isotonic solution of hydrochloric acid, which contains no other cations; we have, therefore,

$$C'_{Cl} = C_o \tag{1}$$

$$C'_B = 0 \tag{2}$$

The alkaline component, however, constitutes a solution of organic matter (mucin, enzymes, etc.) in an isotonic mixture of neutral chloride and bicarbonate.[4] Therefore,

$$C''_{Cl} = C''_{CBl} \tag{3}$$

$$C''_B = C''_{BCl} + C''_{BHCO_3} = C_o \tag{4}$$

Combining Equations 3 and 4, we get

$$C''_{Cl} = C_o - C''_{BHCO_3} \tag{5}$$

Now, gastric juice such as enters into this discussion contains free hydrochloric acid,[5] and therefore all of its alkali and alkaline earth bicarbonate has been converted to neutral chloride by reaction with this acid. Thus

$$C_B = C_{BCl} \tag{6}$$

$$C_{Cl} = C_{BCl} + C_{HCl} \tag{7}$$

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[3] Since the present argument is only a first approximation, it is here assumed that the isosmotic concentration of hydrochloric acid is identical with that for the mixed neutral chlorides and bicarbonates, within the limits of error of this investigation.

[4] Throughout this discussion, minor constituents like the phosphate radical are considered so small as to be negligible in a first approximation.

[5] This mathematical analysis can be developed in such a way as to include gastric juice which possesses an "acid deficit"; i.e., some unneutralized bicarbonate. The resulting complications, however, are hardly justified by the increase in mathematical rigor and universality which would be attained, and so the present discussion is restricted to acidity values greater than zero.

and by combining Equations 6 and 7 we get

$$C_{HCl} = C_{Cl} - C_B \qquad (8)$$

Suppose that the acid and alkaline components are mixed in varying proportions to form a series of gastric juice samples of graded acidities, and let $p = $ the fraction of parietal secretion in any one mixture, and $(1 - p) = $ the fraction of the alkaline component. Then, for each of the resulting mixtures,

$$C_{Cl} = p \times C'_{Cl} + (1 - p) \times C''_{Cl} \qquad (9)$$

$$C_B = p \times C'_B + (1 - p) \times C''_B \qquad (10)$$

Substituting Equations 9 and 10 in Equation 8

$$C_{HCl} = p \times (C'_{Cl} - C'_B) + (1 - p) \times (C''_{Cl} - C''_B) \qquad (11)$$

In order to obtain C_{Cl} as a function of C_{HCl}, let us solve Equations 9 and 11 for p and equate the resulting expressions.

$$\frac{C_{Cl} - C''_{Cl}}{C'_{Cl} - C''_{Cl}} = \frac{C_{HCl} - (C''_{Cl} - C''_B)}{(C'_{Cl} - C'_B) - (C''_{Cl} - C''_B)} \qquad (12)$$

Substituting Equations 5, 1, 4, and 2 in Equation 12, we obtain

$$\frac{C_{Cl} - (C_o - C''_{BHCO_3})}{C_o - (C_o - C''_{BHCO_3})} = \frac{C_{HCl} - (C_o - C''_{BHCO_3} - C_o)}{(C_o - 0) - (C_o - C''_{BHCO_3} - C_o)}$$

and finally, solving for C_{Cl},

$$C_{Cl} = C_{HCl} \times \left[\frac{C''_{BHCO_3}}{C_o + C''_{BHCO_3}} \right] + \left[\frac{C_o{}^2}{C_o + C''_{BHCO_3}} \right] \qquad (13)$$

Similarly, to obtain C_{BCl} as a function of C_{HCl}, let us solve Equations 10 and 11 for p and equate the resulting expressions.

$$\frac{C_B - C''_B}{C'_B - C''_B} = \frac{C_{HCl} - (C''_{Cl} - C''_B)}{(C'_{Cl} - C'_B) - (C''_{Cl} - C''_B)} \qquad (14)$$

Substituting Equations 6, 4, 5, 1, and 2 in Equation 14, we get

$$\frac{C_{BCl} - C_o}{0 - C_o} = \frac{C_{HCl} - (C_o - C''_{BHCO_3} - C_o)}{(C_o - 0) - (C_o - C''_{BHCO_3} - C_o)}$$

and finally, solving for C_{BCl}, we obtain

$$C_{BCl} = C_{HCl} \times \left[\frac{- C_o}{C_o + C''_{BHCO_3}} \right] + \left[\frac{C_o{}^2}{C_o + C''_{BHCO_3}} \right] \qquad (15)$$

Since the bracketed fractions in Equations 13 and 14 are all parameters, these expressions are equations for a straight line in the intercept form, $y = mx + b$. They describe the relations, according to our hypothesis, among the variables C_{Cl}, C_{BCl}, and C_{HCl}. But, the graphs representing the empirical relations among these variables may also be represented by equations in the intercept form as follows:

$$C_{Cl} = m_{Cl} \times C_{HCl} + I_{Cl} \qquad (13, a)$$

$$C_{BCl} = m_{BCl} \times C_{HCl} + I_{BCl} \qquad (15, a)$$

where I_{Cl} and I_{BCl} are the intercepts on the vertical axes, and m_{Cl} and m_{BCl} are the slopes of the respective lines. These slopes are given by the tangents of the angles which the lines make with the horizontal axes (see Columns 4 and 6 of Table I). Thus, Equations 13, a and 15, a, arrived at empirically, are identical in form with Equations 13 and 15 respectively, which were developed solely from theoretical considerations associated with our hypothesis.

It can also be shown that these two pairs of equations possess three specific characteristics in common. From Columns 4 and 6 of Table I we have

$$m_{Cl} - m_{BCl} = 1 \qquad (16)$$

From Equations 13 and 15, these two slopes are given by $\dfrac{C''_{BHCO_3}}{C_o + C''_{BHCO_3}}$ and $\dfrac{- C_o}{C_o + C''_{BHCO_3}}$ respectively. The algebraic difference of these expressions also equals one, which thus establishes a first point of identity. Again, from the empirical equations, we find that the ordinate intercepts are given by

$$I_{Cl} = C_{Cl} - m_{Cl} \times C_{HCl} \qquad (17)$$

$$I_{BCl} = C_{BCl} - m_{BCl} \times C_{HCl} \qquad (18)$$

Subtracting Equation 18 from Equation 17 we get

$$I_{Cl} - I_{BCl} = C_{Cl} - C_{BCl} - (m_{Cl} - m_{BCl}) \times C_{HCl}$$

However, $C_{Cl} = C_{BCl} + C_{HCl}$ and, from Equation 16, $m_{Cl} - m_{BCl} = 1$.
Whence

$$I_{Cl} - I_{BCl} = 0 \quad \text{or} \quad I_{Cl} = I_{BCl} \tag{19}$$

But, the constant terms in Equations 13 and 15 are likewise identical, being equal to $\dfrac{C_o{}^2}{C_o + C''_{BHCO_3}}$. Consequently, the two pairs of equations are seen to possess a second characteristic in common. Finally, it was shown above that the experimental acidity intercept, I_A, is equal to the "isosmotic concentration." From Equation 15, however, we see that when $C_{BCl} = 0$, $C_{HCl} = I_A = C_o$. This establishes a third point of identity between the hypothetical and the empirical relations under consideration.

It is interesting to note that it is possible, from Equations 13, 15, 13, a, and 15, a and the corresponding graphs, to calculate the concentration of chloride in the alkaline constituent of the gastric juice. This has been carried out for the most representative graph (Fig. 3), which is based on a greater number of samples than all the other graphs combined, and a value of 100 mm obtained. Since the actual composition of gastric mucus and the conditions which influence it are unknown, it remains for future investigation to decide whether the agreement of this value with that for blood plasma is significant or merely a coincidence.

Thus, it has been shown that not only are the experimental results in fundamental disagreement with the Rosemann theory, but that they conform in several specific respects to the demands of our own hypothesis. Although this does not constitute absolute proof, it follows with a high degree of probability, therefore, that the chloride ion of gastric pouch juice is derived in part from the parietal fluid and in part from the mucus and other secretions which are present in the pouch. As for the neutral chloride, this comes entirely from the neutral or alkaline components of the mixed juice; the major portion is secreted directly as such whereas the remainder is initially secreted as bicarbonate and subsequently converted to neutral chloride by interaction with the hydrochloric acid already present. The results also support our earlier contention that the normal fluctuations in acidity of pouch juice are not inherent in the parietal secretion but result from neutralization and dilution by mucus, etc.

SUMMARY AND CONCLUSIONS

1. The data here presented confirm the existence of a qualitative parallelism of the curves obtained by plotting the acidity and total chloride concentrations for pure gastric juice against the time. Likewise, the observations suggest an explanation for the occasional failure of other investigators to observe this phenomenon.

2. However, when the total chloride concentration and the acidity are plotted against each other, with the exclusion of time as a factor, the resulting graph is a straight line. This simple mathematical relation between the two variables has been demonstrated with data from a variety of sources.

3. Similarly, a straight line relation has been shown to exist between the neutral chloride concentration and the acidity.

4. A detailed hypothesis has been formulated to account for these quantitative variations in total and neutral chloride concentrations, and also for the different types of acidity-time curves previously reported. This hypothesis assumes that pure gastric juice is a mixture of the parietal secretion with an alkaline fluid which is principally mucous secretion. The parietal secretion is essentially an isotonic solution of hydrochloric acid, and contains no fixed base. The alkaline component is an isotonic solution the principal constituents of which are neutral chlorides and bicarbonates, the concentration of the former being appreciably greater than that of the latter.

5. Finally, it has been shown that the experimental findings support this hypothesis in the following respects: (a) As the acidity diminishes, the total chloride concentration also decreases but at a much lower rate. The Rosemann theory, on the contrary, presumes a concentration of chloride which is constant, independently of variations in acidity. (b) The maximum acidity which can be attained by gastric juice under normal conditions is that of a solution of hydrochloric acid which contains no neutral chloride and is isotonic with mammalian blood. (c) The mathematical equations relating the acidity with the total and neutral chloride concentrations, arrived at theoretically, are identical in certain specific respects with the corresponding equations developed from the experimental graphs.

In conclusion, therefore, it may be stated that the concentra-

tions of total and neutral chloride in pure gastric juice are functions of the acidity in agreement with the above hypothesis.   The process as pictured here, however, is only a first approximation; it now remains to extend it by studying the properties of the several constituent secretions which enter into the mixed gastric juice.

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THE DETERMINATION OF THE ACIDS OF PLANT TISSUE

I. THE DETERMINATION OF NITRIC ACID*

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In the course of an investigation of the organic acids of the leaf of the tobacco plant it was observed that the total acidity of an ether extract, prepared from material that had been acidified to pH 1 with sulfuric acid, was much greater than could be accounted for by the acids that titrate between the limits pH 8.0 and 2.6 according to the technique of Van Slyke and Palmer (1). Quali-tative tests indicated that the ether had extracted the whole of the nitric acid contained in the leaf tissue, and further study showed that a simple and accurate method for the determination of nitric acid can be founded on this observation. Aqueous alkali is added to the extract and the ether is evaporated; a solu-tion is thus obtained, the nitrate content of which may be deter-mined by any suitable method. The details of the procedure, therefore, may be modified according to the special needs of the operator; the essential feature is the extraction of the nitric acid from the tissue by ether.

Adjustment of Tissue Reaction—A preliminary test to determine the quantity of sulfuric acid required to bring the sample of dry tissue to approximately pH 1 must be performed. This is con-veniently accomplished by mixing 0.5 gm. of the material with 1 cc. of 4 N sulfuric acid and diluting with sufficient water to form a thin paste that can be transferred to the electrode vessel; quin-

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hydrone is then added and the reaction is determined. Suitable changes in the quantity of acid employed are made as suggested by the reaction observed. A reaction within the range pH 0.7 to 0.9 has been found satisfactory in our practice. From the preliminary test the quantity of acid needed for the 2.0 gm. sample of tissue used in the nitrate determination is calculated; the materials studied in this laboratory have usually required 3 to 4 cc. of 4 N sulfuric acid for the correct adjustment of a 2 gm. sample.

Ether Extraction—A 2.00 gm. sample of the finely ground, air-dry tissue is mixed, in a 100 cc. beaker, with the required amount of 4 N sulfuric acid until a uniform stiff paste is obtained; 3.5 gm. of pure asbestos fiber are added and thoroughly incorporated, and the mixture is transferred to a 26 × 60 mm. Schleicher and Schüll paper extraction thimble. The transfer is accomplished by the use of a glass funnel about 11 cm. long, the upper part of which is a cylinder 4.5 cm. in diameter, the lower, a cylinder 2 cm. in diameter. This funnel is clamped in position with the wide mouth extending about 1 cm. into the extraction thimble which is held in a wire cage suspended in the mouth of a 400 cc. conical extraction flask. After pushing most of the asbestos into the thimble with a glass rod, the remainder may be brushed from the beaker, rod, and funnel; the glassware is finally wiped off with a small piece of moistened surgical cotton and is thoroughly rinsed into the thimble with alcohol-free ether. The thimble is then plugged with the cotton used in wiping the apparatus and is placed in a siphon tube of the type designed for rubber analysis (Eimer and Amend, catalogue No. 30754). A thin glass rod is thrust into the siphon tube in order to hold the paper thimble away from the glass wall on one side, and the siphon tube is hung, by means of a loop of fine galvanized iron wire, from the spiral metal condenser of the extraction apparatus. The condenser is placed on the conical extraction flask, which contains 150 to 200 cc. of alcohol-free ether, and the extraction is conducted on an electric hot-plate for at least 8 hours. The rate of boiling is important; in our apparatus the ether siphoned back about forty times per hour. Irregularities in boiling are almost entirely eliminated by the use of a few angular quartz pebbles in the flask. Completeness of extraction is confirmed by conducting a diphenylamine test on a concentrated hot water extract of the contents of the thimble.

Analysis of Ether Extract—The ether extract is treated with about 25 cc. of water and 2 drops of phenolphthalein solution; 0.5 N sodium hydroxide is then added with vigorous agitation until the aqueous layer is just alkaline and the ether is distilled off very slowly in order to avoid frothing. The extracts we have investigated have yielded turbid solutions of a brown or green color. The turbidity and practically all of the color can be removed if the solution is acidified to Congo red with sulfuric acid and filtered, but this is unnecessary if the nitrate is to be determined by the acid and iron reduction method that we have employed. The alkaline solution is made to 100 cc. and an aliquot part (10 cc. are usually a convenient amount) is transferred to a 300 cc. Kjeldahl flask together with 2.5 cc. of 18 N sulfuric acid and 0.3 gm. of reduced iron powder. The contents of the flask are boiled gently for 5 minutes, and 20 cc. of water and 10 cc. of ammonia-free saturated sodium hydroxide solution are added. The flask is immediately fitted with a distillation tube of the type employed by Folin and Wright (2); the contents are mixed, and are then distilled over a micro burner into 3 cc. of 0.1 N hydrochloric acid for 5 minutes counted from the time distillate first appears in the vapor tube. The distillate is transferred to a 100 cc. flask, diluted to about 60 cc., and treated with 10 cc. of Nessler's reagent. It is then made to volume and the color is read against a standard solution of ammonium sulfate (0.3 to 1.5 mg. of nitrogen). A correction must always be applied for the nitrogen content of the reduced iron powder. This is determined by boiling 3.0 gm. of the powder with 50 cc. of 10 per cent sulfuric acid for 5 minutes; the ammonia produced is then liberated, distilled into 0.1 N acid, and titrated. The quantity found divided by 10 gives the correction that must be subtracted to allow for the ammonia that arises from the 0.3 gm. of iron powder employed in the nitrate determination.

When dealing with a plant tissue that is low in nitrate, it is desirable to carry out a blank determination on an equal aliquot part of the alkaline extract. This is conducted in exactly the same manner as the determination except that the iron powder is omitted. The distillate, after treatment with Nessler's solution, is made to 25 cc. and is read against a 0.05 to 0.10 mg. standard. In practice, however, this blank determination is so small that,

except when dealing with tissues of low nitrate content, it may be disregarded.

Tests of the Method—In order to demonstrate the quantitative extraction of nitric acid by ether measured quantities of standard solutions of potassium nitrate were absorbed on asbestos, a sufficient quantity of 4 N sulfuric acid was added, and the mixture was transferred to the extraction thimble. In cases where more than 5 cc. of fluid were used the mixture on the asbestos was dried in a vacuum desiccator until it could be conveniently handled. Under these conditions the recovery of from 1.00 to 20.00 mg. of nitrate

TABLE I

Recovery of Nitrate Added to Tobacco Leaf Tissue

The figures are percentages of the weight of the dry tissue.

Nitrate N				Difference (4) − (2)
Originally present (1)	Added (2)	Found (3)	Recovered (4)	
0.24	0.50	0.75	0.51	+0.01
0.23	0.50	0.73	0.50	0.00
0.24	0.50	0.73	0.49	−0.01
0.24	0.50	0.70	0.46	−0.04
0.27	0.20	0.46	0.19	−0.01
0.27	0.29	0.55	0.28	−0.01
0.27	0.39	0.64	0.37	−0.02
1.07	0.30	1.38	0.31	+0.01
1.07	0.30	1.35	0.28	−0.02
1.05	0.30	1.34	0.29	−0.01
1.05	0.30	1.34	0.29	−0.01

nitrogen (nineteen experiments) averaged 98.75 per cent, the lowest recovery being 97.7 per cent (two experiments) and the highest 101 per cent (one experiment).

The recovery of nitrate added to tobacco leaf tissue was almost equally satisfactory. In eleven experiments the average recovery of the added nitrate was 97.1 per cent. The data for these experiments, expressed as percentage of nitrate in, or added to, the tissue, are shown in Table I.

In all of these preliminary experiments the extraction with ether was allowed to continue for 15 to 20 hours; color tests on the residue had shown this to be adequate. A series of experiments

in which the time of extraction was varied showed that the diphenylamine reaction given by the residue was negative after 6 hours extraction and was very faint or doubtful after 5 hours. It must be emphasized, however, that the time of extraction depends on the efficiency of the ether extraction device employed; with our apparatus, which siphoned at the rate of approximately forty times per hour, extractions were invariably complete in 7 to 8 hours.

TABLE II

Comparative Analyses by Ether Extraction Method and by Other Methods

Sample No.	Nitrate N		
	Vickery and Pucher method	Jones method*	Ether extraction method
	per cent	*per cent*	*per cent*
582		0.58	0.76
587		0.72	0.86
590		0.78	0.85
591		0.70	0.955
592		0.60	0.83
594		0.80	0.77
612		0.12	0.36
624		0.00	0.025
A	0.79		0.75
B	0.74		0.77
C	0.63		0.615
D	0.78		0.75
E	0.60		0.63

* Data kindly supplied by the Department of Analytical Chemistry of this Station.

Comparison of Results of Ether Extraction Method with Other Methods—Analyses for nitrate of a number of samples of tobacco leaf tissue in which the Jones method (3), and the modification of this method suggested by Vickery and Pucher (4), were employed are shown in Table II; each figure represents the average of two or more determinations. The agreement between the results of the new method and those of the modified Jones method is satisfactory. The original Jones method is less satisfactory, however, but it must be remembered that this method is subject to an error

when applied to tobacco leaf owing to the volatility of nicotine. A discussion of this point is given by Vickery and Pucher (4).

Sample 624 is of particular interest because no nitrate could be detected in it by the Jones method. A hot water extract of this material gave no perceptible reaction with diphenylamine; nevertheless the ether extract obtained from it responded positively to this reagent. The sensitivity of the diphenylamine reagent was therefore investigated. A solution that contained 0.01 mg. of nitrate nitrogen per cc. as potassium nitrate gave a faintly positive test with our reagent but, if sucrose was added to this solution, the brown color produced by the action of the sulfuric acid during the test entirely masked the blue color. A water extract of tobacco leaf is highly colored and contains substances that are darkened by sulfuric acid; the diphenylamine reaction is therefore relatively insensitive when applied to it. On the other hand, the solution of the nitrate obtained by extraction with ether contains very little organic matter that interferes with the diphenylamine reaction.

As a further demonstration that Sample 624 does, in fact, contain nitrate, 1.593 gm. of the dry material were extracted with ether and the alkaline solution derived from the ether extract was neutralized, filtered, and treated with nitron acetate. The amorphous precipitate produced was recrystallized. The product showed typical crystals of nitron nitrate and weighed 7.4 mg., the equivalent of 0.017 per cent of nitrate nitrogen in the tissue.

According to Sessions and Shive (5) the bulbs of paper-white narcissus are free from nitrate. In order to test the present method on this tissue the bulbs were stripped of their outer scales, sliced, and dried, and 2.0 gm. samples of the finely ground material were extracted with ether as described. The extract gave no test for nitrate with diphenylamine. The analyses of this extract were conducted with careful attention to the blank determinations. In four experiments an average of 0.018 per cent of nitrogen was found as ammonia after reduction with sulfuric acid and iron. The four blank experiments without iron gave an average of 0.014 per cent of nitrogen as ammonia, while the blank on the iron powder used amounted to the equivalent of 0.005 per cent.

A sample of tobacco seed was also analyzed for nitrate. The ether extracts obtained in three experiments each gave a doubtfully

positive test with diphenylamine; on reduction with sulfuric acid and iron powder the average quantity of ammonia corresponded to 0.040 per cent of nitrate nitrogen in the tissue. The blank corresponded to 0.025 per cent and the blank on the iron powder to 0.014 per cent. Tobacco seed therefore apparently contains a trace of nitrate. These results illustrate the statement already made that blank determinations are necessary in the present method when dealing with tissues that contain low proportions of nitrate.

Gravimetric Determination of Nitrate—The ether extraction method furnishes a solution in which nitrate can be determined as nitron nitrate with considerable accuracy. In certain cases this method may be desirable. For this purpose an aliquot part of the alkaline solution that contains 2 to 5 mg. of nitrate nitrogen is acidified to Congo red with dilute sulfuric acid, is allowed to stand until the precipitate flocculates, and is then filtered. The precipitate is washed with a little water and discarded. The filtrate (not more than 35 cc.) is heated to boiling and 2 to 3 cc. of 10 per cent solution of nitron acetate are added. After being chilled overnight the precipitate is filtered on a Gooch crucible fitted with a disc of filter paper. The volume of the filtrate is recorded and the precipitate is then washed with 10 to 15 cc. of ice water. The crude nitron nitrate is transferred to a small beaker, dissolved in a minimal quantity of boiling water, and 2 to 3 drops of nitron acetate solution are added; after being chilled overnight the recrystallized nitron nitrate is filtered on a weighed Gooch crucible, the volume of the filtrate being recorded as before. The product is then washed with a little ice water, is dried at 100°, and weighed. The nitrate nitrogen in the aliquot taken is calculated from the equation, 0.03734 (mg. of nitron nitrate + 0.099 (cc. of total filtrate)) = mg. of nitrate nitrogen.

The recovery of nitrate from potassium nitrate solutions by this procedure was satisfactory for quantities between 2.5 and 8 mg., the average of six experiments being 102 per cent. A series of determinations on the samples of tobacco previously mentioned is shown in Table III (Columns 2 and 3). The necessity of recrystallization of the nitron nitrate is obvious, from a consideration of the melting points of the preparations. The material obtained in the first precipitates melted, as a rule, somewhat slug-

gishly over the range 261–263° uncorrected. Under the micro-
scope it was not homogeneous. After recrystallization the
preparations were finely crystallized in long prisms and were
homogeneous. They invariably melted sharply at 265–265.5°
when observed with the same long stem thermometer.

Column 4 of Table III gives the results of the estimations of
nitrate in the ether extract carried out by the iron reduction
method. These figures are corrected for the ammonia contributed

TABLE III

*Comparison of Nitrate Nitrogen in Tobacco Leaf As Determined Gravi-
metrically with Determinations by Ether Extraction Method*

Sample No. (1)	Nitron nitrate		Ether extraction*	
	First precipitate (2)	Second precipitate (3)	No blank (4)	Corrected for blank (5)
	per cent	per cent	per cent	per cent
587	0.91	0.88	0.89	0.86
590	0.98	0.94	0.88	0.85
591	1.01	0.97	0.98	0.95
592	0.90	0.85	0.86	0.83
594	0.94	0.82	0.81	0.78
624	0.06	0.02	0.06	0.03
A	0.93	0.86	0.78	0.75
B	0.88	0.82	0.80	0.77
C	0.73	0.66	0.64	0.61
D	0.89	0.84	0.78	0.75
E	0.74	0.71	0.65	0.63
F	1.11	1.07	1.07	1.02
Watercress	0.655	0.605	0.59	0.55

* The figures in these columns are all corrected for the small amount of
ammonia derived from the iron used for reduction.

by the iron powder but are not corrected for the blank determina-
tion in which iron powder was omitted. Column 5 gives the re-
sults when both blank corrections are applied. It is clear that the
gravimetric method gives, in general, slightly higher results than
the reduction method, but the agreement between the two
methods is entirely satisfactory. The data in Columns 4 and 5
also illustrate the statement that the blank determination on the
ether extract may be omitted without introducing serious error

into the final result in cases where the proportion of nitrate is fairly high.

Determination of Nitrate in Aqueous Extracts—The discussion of the ether extraction method for the determination of nitrate has hitherto been confined to the examination of samples of dry tissue. The method can be applied, however, to aqueous extracts provided these are concentrated to a point where, after the addition of asbestos, a mass is obtained that can be transferred to the extraction thimble. Experiment showed that extracts that had been previously acidified to pH 1 could not be concentrated on a water bath without loss of nitric acid. This observation confirms that of Nelson, Levine, and Buchanan (6). It is therefore necessary to conduct the evaporation at a neutral reaction. An aliquot part of the extract equivalent to approximately 2 gm. of dry tissue is made neutral to Congo red and is evaporated on a steam bath to a sirup; it must not be evaporated to dryness. After being cooled the sirup is mixed with the amount of 4 N sulfuric acid required to produce a reaction of pH 0.7 to 0.9 as determined by a separate experiment. The asbestos (3.5 gm.) is then thoroughly incorporated and, if the mass is still too moist for transfer, it is dried in a vacuum desiccator for a short time.

When treated in this way an extract of tobacco leaf was found to contain 7.53 mg. of nitrate nitrogen per 100 cc. (average of six experiments). The nitrate content determined by the nitron method was 7.65 mg. per 100 cc. and the result of the modified Jones method was 7.45 mg. To samples of this extract potassium nitrate equivalent to 6.00 mg. of nitrogen was added and the procedure above described was applied. The average recovery was 5.90 mg. (six experiments). As further evidence of the completeness with which nitrate can be recovered eight samples of an extract of paper-white narcissus bulbs, previously shown to contain no nitrate, were treated with amounts of from 1.0 to 22 mg. of nitrate nitrogen, and calculated to be the equivalent of a nitrate nitrogen content of 0.05 to 1.09 per cent of the dry tissue. The recovery of the 1.0 mg. quantities was somewhat high, 1.20 and 1.19 mg. being found; the recovery of 5 to 22 mg. quantities averaged 98.8 per cent.

Nitrogen of the Ether Extract—The ether extract of tobacco leaf tissue, obtained under the conditions described above, contains as

a rule about 80 per cent of its nitrogen in the form of nitrate; in a series of eight samples the amount varied between 77 and 87 per cent. Extracts from tobacco of low nitrate content, or from the tissues of other plants, may contain considerably lower proportions of the nitrogen in this form. Nevertheless, the ether extract provides a fraction that contains all of the nitrate of the tissue, and the total quantity of nitrogen in other forms is usually very small.

When the green or brown, turbid alkaline solutions containing the substances extracted by ether are acidified to Congo red with sulfuric acid, a flocculent precipitate separates, the filtrate from which is usually colorless. The precipitate contains nitrogen, the average result obtained from a number of samples indicating an order of magnitude of one-quarter of the non-nitrate nitrogen of the extract. The filtrate usually contains a detectable quantity of ammonia and, in addition, small amounts of substances that yield ammonia on mild acid hydrolysis. Nicotine is invariably absent. Preliminary experiments only have been carried out on this fraction but, the suggestion already obtained that ether-soluble amides may be present appears to be a matter of some importance. Substances of similar behavior were detected in extracts prepared from watercress leaves, from narcissus bulbs, and from tobacco seed. Further investigation of this is planned.

SUMMARY

A method for the determination of the nitrate content of plant tissue is described, which depends upon the observation that nitric acid is quantitatively extracted by ether from tissue that has previously been treated with sufficient sulfuric acid to bring it to a reaction approximating pH 1. The acidic substances in the ether extract are transferred to aqueous alkali and the nitrate in this solution is determined either by reduction to ammonia, by boiling with acid and iron powder followed by distillation and estimation by Nessler's method, or the nitrate is determined gravimetrically as nitron nitrate. There is no doubt that other methods could equally well be applied.

The ether extract from the tissue contains a small proportion (20 to 30 per cent) of its nitrogen in forms other than nitrate. In particular it contains a measurable proportion of ammonia and

also of substances that yield ammonia on mild acid hydrolysis. Although the presence of these renders it necessary to conduct blank experiments when the nitrate is determined by reduction with acid and iron, the magnitude of the blank is small and, in cases in which the nitrate content of the tissue is high, the blank determination may be neglected without serious error.

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THE CHEMISTRY OF THE LIPIDS OF TUBERCLE BACILLI

XXVII. THE COMPOSITION OF THE PHOSPHATIDE FRACTION OF THE BACILLUS LEPRÆ*

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INTRODUCTION

In the first chemical analysis of the leprosy bacillus by Gurd and Denis (1), it was reported that the ether-soluble constituents contained phosphorus, and hence these authors suggested that lecithin was present, but beyond this mere suggestion nothing has been known regarding the nature of the phosphatide of *Bacillus lepræ*. As described in a former paper (2), we were able to secure about 100 gm. of phosphatide from the alcohol-ether extract of a very large quantity of *Bacillus lepræ*. The present report deals with the chemical composition of the phosphatide and with the identification of the principal cleavage products which are liberated when the substance is hydrolyzed or saponified.

It is a matter of interest to us that the present work brings to completion the analyses of the phosphatides from five standard strains of acid-fast bacilli; namely, the human (3), avian (4), and bovine (5) tubercle bacilli, the timothy bacillus (6), and the leprosy bacillus. All of these bacilli contain phosphatides which are similar in properties and in chemical composition, but quantitatively the amount of phosphatide obtained from the different strains of bacteria varies greatly. All of the phosphatides from

the acid-fast bacteria possess unusually interesting chemical and biological properties.

The biological reactions (7) have been studied in Dr. Sabin's laboratory at the Rockefeller Institute, and it has been found that the phosphatides exert, when injected intraperitoneally into healthy animals, a pronounced stimulating effect on the proliferation of monocytes, epithelioid cells, and giant cells, which leads to the formation of tubercular tissue.   While this effect is general, there has been observed a notable variation in the intensity of the reaction with different bacillary phosphatides, the preparation from the human tubercle bacillus showing the greatest effect.

By reason of the biological activity of the phosphatides, we have been especially interested in determining the chemical compounds which are liberated when the phosphatides are hydrolyzed.   While it has not been possible to identify every compound which is formed, it may be stated that the principal cleavage products have been isolated and identified.   The fatty acids in all of these cases consist of (a) solid saturated acid, usually palmitic acid, (b) liquid unsaturated acid, presumably oleic acid, because on catalytic reduction stearic acid is formed, (c) peculiar new liquid saturated fatty acids of high molecular weight.   The water-soluble components, after complete hydrolysis, have been found to consist of (a) glycerophosphoric acid, (b) inosite, (c) mannose, and (d) some other reducing hexose sugar.

In the analysis of the phosphatide from the leprosy bacillus, we have encountered all of these cleavage products, and, in addition, we have found some other compounds.   Among the ether-soluble constituents, a wax-like substance of high molecular weight and acidic properties was found which in many respects was similar to the so called unsaponifiable wax (8) from the human tubercle bacillus, although it differed from the latter substance in that it was optically active and in that it was also unsaturated.   The solid saturated fatty acid fraction contained, in addition to palmitic acid, a small amount of a higher acid, but the amount of this acid was too small to permit of its identification.   The unsaturated acid fraction gave on catalytic reduction a mixture of palmitic and stearic acids.   These two acids were separated and identified.   The liquid saturated fatty acid was very similar in its properties and composition to tuberculostearic acid (9).

The phosphatide from the leprosy bacillus is very stable and it was only partly hydrolyzed after it had been refluxed for 12 hours with dilute sulfuric acid. It is more readily saponified when refluxed with dilute alcoholic potassium hydroxide. The cleavage products, after alkaline saponification, are separated sharply into alcohol-soluble and alcohol-insoluble products. The alcoholic solution contains the ether-soluble constituents, such as wax and fatty acids, in the form of soaps. The alcohol-insoluble fraction is soluble in water and consists of glycerophosphate and a complex polysaccharide. The mechanical separation of these two main fractions is complete and very convenient. The separation of the water-soluble components, the glycerophosphoric acid, and the polysaccharide was accomplished by means of neutral lead acetate which gives a water-insoluble lead glycerophosphate, while the polysaccharide remains in solution and can be isolated from the filtrate. By precipitating the concentrated aqueous solution of the polysaccharide with alcohol, the substance is obtained as a snow-white amorphous powder which contains phosphorus in organic combination. When the polysaccharide is refluxed with dilute sulfuric acid, it becomes completely hydrolyzed in the course of 3 to 4 hours and yields inorganic phosphoric acid together with about 2 parts of mannose, 1 part of inosite, and 1 part of a reducing hexose sugar which gives glucosazone on treatment with phenylhydrazine hydrochloride and sodium acetate.

All of the lipid fractions from the leprosy bacillus are highly pigmented and the phosphatide was of a bright reddish color. We attempted to isolate the pigment in the present analysis by extracting with ether the red-colored soap solution which was obtained after the phosphatide had been saponified with alcoholic potassium hydroxide. The ethereal extract was of reddish color and a red oily residue remained when the solvent was evaporated. The color was not affected by either acid or alkali. The pigment was readily soluble in ether, chloroform, and methyl alcohol, but no crystalline substance could be obtained from these solutions The methyl alcoholic solution of the pigment gradually faded to a pale yellow color, and when the solvent was allowed to evaporate there remained only a small amount of a yellow oil.

The preparation of the phosphatide was described briefly in Paper XXVI (2) of this series.   The substance had been isolated from an alcohol-ether extract of 3000 cultures of *Bacillus lepræ*. The organism had been cultivated on the Long (10) synthetic medium in the Mulford Biological Laboratories, Sharp and Dohme, at Glenolden, Pennsylvania.

The crude phosphatide, after it had been precipitated repeatedly from ethereal solution with acetone, formed an amorphous powder of a bright reddish color and possessed the properties mentioned below.   When the phosphatide was rubbed up with water, it formed a colloidal solution which was distinctly acid in reaction to litmus.   The colloidal solution was precipitated or coagulated by the addition of acids or salts.   When the acidified solution was heated to boiling, a dense coagulum formed which was disintegrated very slowly and incompletely on continued boiling under a reflux condenser.   An aqueous suspension of the phosphatide, when boiled with Fehling's solution, gave no reduction, but, after the suspension had been boiled for at least 10 minutes with dilute acid, cooled, neutralized, and filtered, the filtrate reduced Fehling's solution.   It is evident, therefore, that the phosphatide from the leprosy bacillus is very stable and that it contains a polysaccharide which yields reducing sugars on hydrolysis.   In this respect the substance is similar to the phosphatides which have been isolated from other acid-fast bacteria, but the substance from the leprosy bacillus appears to be more stable.

The leprosy bacillus is highly chromogenic and all of the lipid fractions were of a deep red color.   The pigment adhering to the phosphatide could not be removed by precipitation from ethereal solution with acetone, but by precipitating the substance a few times from chloroform solution with methyl alcohol, a nearly white or faintly straw-colored amorphous powder was obtained.   The purified preparation melted with decomposition at 231°.   It contained 1.75 per cent phosphorus and only a trace of nitrogen.

A series of biological experiments are being conducted in Dr. Sabin's laboratory at the Rockefeller Institute to determine the cellular reactions of the purified preparation.   The results of this investigation will be reported separately by Dr. Sabin and her collaborators.

The chemical analyses reported in this paper were made on the crude preparation which had been precipitated with acetone from ethereal solution. In order to prevent as much as possible the oxidation of unsaturated compounds, all operations were conducted in an atmosphere of carbon dioxide or nitrogen until the unsaturated fatty acids had been reduced. All solvents employed had been freshly distilled and the alcohol had been distilled over potassium hydroxide.

Acid Hydrolysis of Phosphatide

In a preliminary experiment an attempt was made to hydrolyze the phosphatide with boiling 5 per cent sulfuric acid, but it was found that the substance was so stable that this method was unsatisfactory. Ordinary phosphatides are usually easily hydrolyzed in a few hours when refluxed with dilute sulfuric acid; the coagulum which forms at first being gradually converted into a clear oily layer of fatty acids. In the present case 15 gm. of the phosphatide were rubbed into a colloidal suspension in 400 cc. of water, and to this mixture were added 100 cc. of 25 per cent sulfuric acid. A heavy coagulum separated on heating to boiling and this material was only partly disintegrated after the mixture had been refluxed for 12 hours. The solution was therefore cooled and the insoluble matter, which formed a hard cake, was removed and washed with water. The dilute acid solution and washings were extracted with ether and the ethereal solution, after it had been washed with water, was concentrated to dryness. The slight residue which was thus obtained was combined with the partly hydrolyzed phosphatide.

The aqueous acid solution was examined for carbohydrates by the methods described in Paper XXV (6). The solution was found to contain a small amount of phosphoric acid, but no glycerophosphoric acid could be isolated. The mannose phenylhydrazone which was next isolated weighed 1.18 gm., corresponding to 0.78 gm. of mannose. The hydrazone, after it had been recrystallized from hot 60 per cent alcohol, was obtained in the typical plate-shaped crystals and melted with decomposition at 195–196°. The filtrate from the crude mannose phenylhydrazone yielded only 0.1 gm. of inosite. The inosite was identified by its crystal form, the positive Scherer reaction, and the melting point, 225–226°.

The partly hydrolyzed phosphatide was refluxed for 4 hours with 500 cc. of 1 per cent alcoholic potassium hydroxide. The substance did not dissolve completely; some oily drops remained suspended in hot solution, and an insoluble sticky mass remained on the bottom of the flask. The insoluble matter solidified when the mixture was cooled to room temperature. The alcoholic solution was filtered off and the insoluble matter was washed thoroughly with alcohol.

The alcohol-insoluble matter was partly soluble in water. By treatment with warm ether and chloroform a small amount of wax-like material was obtained, but this fraction, which weighed 1.49 gm., corresponding to 9.8 per cent of the phosphatide, was not further examined. The water-soluble material was boiled for several hours with dilute sulfuric acid and the solution was examined by the usual procedure for carbohydrates, but it contained only a trace of reducing sugar and no definite substance could be isolated from this mixture. Evidently the carbohydrate complex had been decomposed by the successive treatments with dilute sulfuric acid and alcoholic potassium hydroxide. In any event, the only water-soluble constituents that could be identified were obtained from the acid hydrolysate and consisted of mannose phenylhydrazone and inosite.

Examination of Fatty Acids

The fatty acids contained in the alkaline alcoholic solution were isolated and formed a semicrystalline solid which weighed 8.6794 gm., which is equal to 57.8 per cent of the phosphatide. The crude acids had an iodine number of 32.6. The acids were separated by the methods described in Paper XIX (5) into (a) solid saturated acid, (b) liquid acids, (c) reduced acids, (d) liquid saturated fatty acid.

The solid saturated fatty acid weighed 2.7906 gm. It was recrystallized twice from methyl alcohol yielding thin, irregular, snow-white plates, melting point 60.5–61.5°. A second fraction, obtained on concentrating the mother liquor, melted at 61–62°. The molecular weight determined by titration was 258.8. These values would indicate that the solid saturated fatty acid consisted principally of palmitic acid.

Liquid Fatty Acids

The liquid fatty acids weighed 4.4728 gm. and the iodine number was 55. The mixture was reduced (11) and the reduction product was separated by means of the lead soap-ether procedure.

The reduced acid was a white crystalline solid which weighed 2.0727 gm. After the acid had been recrystallized twice from methyl alcohol it melted at 63–64°, but two further crystallizations from acetone raised the melting point to 68–69°. The molecular weight, determined by titration, was 282.8. These values would indicate that stearic acid had been formed on reduction, but a lower acid was undoubtedly also present. As will be shown later, the reduced acid was a mixture of palmitic acid and stearic acid.

Liquid Saturated Fatty Acid

The liquid saturated fatty acid was isolated from the ether-soluble lead salts after removing the reduced acid. The ethereal solution of the acid, which was of an orange-red color, was decolorized with norit. The solvent was evaporated, when a nearly colorless oil was obtained which weighed 2.0321 gm. The acid will be described more fully later on, but it may be stated here that it was optically inactive and in chloroform solution it did not absorb any bromine.

Alkaline Saponification of Phosphatide

The phosphatide, 25 gm., was refluxed with 1 liter of 1 per cent alcoholic potassium hydroxide in an atmosphere of nitrogen for 8 hours. The substance did not dissolve completely, a considerable amount of insoluble matter remaining on the bottom and sides of the flask. The hot alcoholic solution, which was clear and of a deep red color, was decanted and the flask with the insoluble residue was rinsed repeatedly with boiling alcohol.

The insoluble residue was reserved for the investigation of the polysaccharide component.

The alcoholic solution was concentrated by distillation to a volume of about 300 cc. and mixed with an equal volume of hot water, giving a perfectly clear red-colored solution. On standing overnight, a voluminous amorphous precipitate had separated which was filtered off on a Buchner funnel and washed with cold 50 per cent alcohol. After this substance had been dried in a vacuum

desiccator, it formed a red brittle wax-like compound weighing 2 gm.   It was reserved for examination as will be described later.

The filtrate was largely diluted with water and extracted four times with large volumes of ether in order to remove the pigment and any unsaponifiable matter.

Pigment and Unsaponifiable Matter

The ethereal extract was washed with water, dried over sodium sulfate, filtered, and the ether distilled.   The residue was a deep red oil which, after drying *in vacuo*, weighed 0.80 gm.   The substance was easily soluble in chloroform or in ether.   The addition of methyl alcohol to these solutions caused an amorphous white precipitate.   The latter was filtered off and an attempt was made to separate the pigment from the alcoholic solution, but without success.   When the methyl alcoholic solution was allowed to stand, the color gradually faded and, on evaporating the solution, a yellowish oily substance remained.   In these experiments no information could be obtained concerning the nature of the pigment.

Separation of Fatty Acids

The alkaline solution from which most of the pigment had been extracted, as mentioned above, was acidified with hydrochloric acid and the fatty acids which separated were extracted with ether. The ethereal solution was washed with water until it was freed from hydrochloric acid and was then dried with sodium sulfate.   The solution was filtered and the sodium sulfate washed with ether. The ether was distilled off and the residue, consisting of the crude fatty acids, was dried *in vacuo*.   A reddish crystalline solid was obtained which weighed 13.7 gm., which is equal to 54.8 per cent of the phosphatide.

The fatty acids were separated into solid and liquid acids by means of the lead soap-ether treatment in the manner already described.   The liquid acids were subjected to catalytic reduction (11) and the lead soap-ether treatment was repeated in order to separate the reduced acid.   The following amounts of the acid fractions were obtained: solid saturated acid, 6.2 gm.; liquid acid, 7.3 gm.; reduced acid, 4.4 gm.; and liquid saturated fatty acid, 2.7 gm.

Solid Saturated Fatty Acid

In the first analysis, as has been stated previously, the constants of the purified solid saturated acid indicated that it consisted principally of palmitic acid. The more careful examination of this fraction in the second analysis gave evidence of the presence of a small amount of some higher fatty acid. The solid saturated acid, after having been recrystallized five times from methyl alcohol and acetone, melted at 62–63°, and the molecular weight, determined by titration, was found to be 269 and 268.9. A second fraction of the acid isolated from the mother liquors melted at 60–61° and the molecular weight was 258.8.

The neutral soap solutions from the titrations of the top fraction on standing deposited a notable amount of a white precipitate, while the soap solution of the second fraction remained perfectly clear. Since potassium palmitate is quite easily soluble in alcohol, it seemed probable that the precipitate which was observed was caused by some higher fatty acid.

The top fraction of the acid was therefore dissolved in alcohol and the solution neutralized to phenolphthalein with alcoholic potassium hydroxide. A precipitate which separated on cooling was filtered off, washed with alcohol, and redissolved in dilute alcohol. The solution was diluted with water, acidified with hydrochloric acid, and the fatty acid which separated was filtered off, washed free of hydrochloric acid with water, and dried *in vacuo*. The dried substance, which weighed 1.4 gm., was reprecipitated five times from acetone. It was easily soluble in hot acetone and separated on cooling in colorless globular particles which showed no crystalline structure. The purified substance weighed 0.2 gm. and melted at 65°.

Titration—0.2010 gm. of substance dissolved in neutral alcohol, with phenolphthalein as indicator, required 4.30 cc. of 0.1 N alcoholic KOH. Found: mol. wt., 467.

Microanalysis—Found, C 78.28, 78.54, H 12.40, 12.53

It is hoped that a larger quantity of this acid will be found in some of the other lipid fractions from the leprosy bacillus so that we may be able to investigate this substance further.

Purification of Palmitic Acid

The filtrate from the above mentioned insoluble potassium soap was diluted with water, acidified with hydrochloric acid, and the fatty acid which separated was filtered off, washed with water, and dried *in vacuo*. A small amount of the higher acid was apparently still present because when the acid was dissolved in methyl alcohol a slight cloudiness was observed which disappeared as the solution was heated, but as the solution cooled a faint turbidity appeared before the actual crystallization of the palmitic acid began. In order to remove this contaminant, the acid was dissolved in a sufficiently large volume of methyl alcohol to hold all of the palmitic acid in solution when cooled to room temperature. A small amount of an amorphous precipitate separated and was removed by filtration. The filtrate was concentrated to a small volume and cooled, when irregular colorless plates separated. The crystals were filtered off, redissolved in methyl alcohol, and the solution was allowed to stand at room temperature as at first. A slight amorphous precipitate was filtered off and these operations were repeated until no further precipitate formed. The purified palmitic acid which was finally isolated weighed 2.3 gm. It was a snow-white product and crystallized in small colorless irregular plates. The acid melted at 62–63°, solidified at 60–59°, and remelted at 63°.

Titration—0.5391 gm. of substance dissolved in neutral alcohol, with phenolphthalein as indicator, required 20.89 cc. of 0.1 N alcoholic KOH. Found: mol. wt., 258.

Microanalysis—Found, C 75.14, 75.46, H 12.40, 12.57; calculated for $C_{16}H_{32}O_2$ (256), C 75.00, H 12.50

A second fraction of the acid was isolated from the mother liquors and recrystallized from methyl alcohol. The snow-white product melted at 62–63°, solidified at 60–59°, and remelted at 62–63°. The molecular weight, determined by titration, was 253.6.

It is evident from the data presented above that the solid saturated fatty acid consisted principally of palmitic acid, but a very small quantity of a higher acid was also present. The nature of this higher acid could not be determined very accurately owing to the small amount that was obtained. It is a curious fact that

the presence of this higher acid had no effect on the melting point of the palmitic acid.

The Reduced Acid

The reduced acid, which weighed 4.4 gm., was recrystallized twice from methyl alcohol and three times from acetone, when 1.2 gm. of snow-white thin irregular plate-shaped crystals were obtained. The acid melted at 71–72°, solidified at 68–69°, and remelted at 71–71.5°. There was no depression of the melting point when some of the acid was mixed with pure stearic acid which melted at 71–72°.

Titration—0.7538 gm. of substance dissolved in neutral alcohol, with phenolphthalein as indicator, required 26.51 cc. of 0.1 N alcoholic KOH. Found: mol. wt., 284.4.

Microanalysis—Found, C 76.26, 76.18, H 12.66, 12.63; calculated for $C_{18}H_{36}O_2$ (284), C 76.05, H 12.67

The constants and analytical data indicate that this top fraction of the reduced acid was stearic acid.

The mother liquors from the recrystallizations mentioned above were concentrated and a second fraction of the acid was isolated. This fraction melted at 61–62° and the molecular weight was 271. Further concentration of the mother liquor gave a third fraction which melted at 61–62° and the molecular weight was 263.5. It seemed evident therefore that some acid lower than stearic acid was present. Fractions 2 and 3, together with the material obtained by evaporating the final mother liquor to dryness, were united giving 3.2 gm. of acid. The acid was converted into the methyl ester and the latter distilled in a high vacuum. The first fraction of the ester, about 1.5 gm., which distilled at 100–103° at a pressure of 0.005 mm. with the bath at 120°, was collected. The ester which melted at 28–29° was saponified and the free acid was isolated and twice recrystallized from methyl alcohol. Snow-white thin irregular plate-shaped crystals were obtained which weighed 0.8 gm. The acid melted at 62–63°, solidified at 59.5°, and remelted at 62–63°.

Titration—0.3579 gm. of substance dissolved in neutral alcohol, with phenolphthalein as indicator, required 13.96 cc. of 0.1 N alcoholic KOH. Found: mol. wt., 256. Calculated for palmitic acid, $C_{16}H_{32}O_2$, mol. wt., 256.

The data indicate that this fraction consisted of palmitic acid, and it must have been formed by reduction of an unsaturated C_{16} acid. This is the first time that we have found palmitic acid in the reduced acid fraction from the phosphatides of the acid-fast bacteria. While we have no direct evidence regarding the degree of unsaturation of the unsaturated fatty acids, it seems likely that only oleic acid and palmitoleic acid were present. The original liquid fatty acids weighed 7.3 gm. and the iodine number was 55. The amount of oleic acid which would correspond to this iodine number would be 4.4 gm. The saturated fatty acids obtained after reduction weighed 4.4 gm. When we consider how difficult it is to separate a mixture of fatty acids quantitatively, it would seem that the agreement of the figures mentioned above makes it reasonably certain that only unsaturated fatty acids with one double bond were present.

Liquid Saturated Fatty Acid

The liquid saturated fatty acid obtained from the ether-soluble portion of the lead salt after separating the reduced acid was a nearly colorless oil which weighed 2.2 gm. It was combined with 1.9 gm. of the similar fraction which had been isolated in the first analysis. The acid was again converted into the lead soap and the latter was dried and treated with ether. A slight amount of insoluble lead soap was filtered off and the free acid was again isolated. The acid was esterified in methyl alcohol and the methyl ester was distilled at a pressure of 0.002 mm. Distillation commenced when the temperature of the bath was 130° and the bath was kept between 130–135°. The temperature of the distillate was between 115–118°. At the end, the distilling flask was empty. The distilled ester was a colorless mobile oil and weighed 3.9 gm. It solidified when cooled to −14° and liquefied in ice water. In ethereal solution the substance was optically inactive. The refractive index at 25° was 1.4425 and the density at 20° was 0.8673.

Analysis—0.1486 gm. substance: 0.1734 gm. H_2O and 0.4171 gm. CO_2
$C_{19}H_{38}O_2$ (298).   Calculated.   C 76.51, H 12.75
                           Found.   " 76.55, " 13.05

The analytical values agree with the calculated composition of a methyl ester of a saturated C_{18} acid.

The ester was saponified and the free acid isolated and dried *in vacuo*. The acid was a mobile colorless oil and weighed 3.4 gm. It solidified when cooled in ice water and liquefied at 14–15°. In chloroform solution it did not decolorize bromine.

Titration—0.3886 gm., 0.5301 gm., 0.3271 gm. of substance dissolved in neutral alcohol, with phenolphthalein as indicator, required 13.13 cc., 18.02 cc., and 11.12 cc. of 0.1 N alcoholic KOH. Found: mol. wt., 295, 294, and 294.

Analysis—0.1392 gm. substance: 0.1593 gm. H_2O and 0.3887 gm. CO_2
$C_{18}H_{36}O_2$ (284). Calculated. C 76.05, H 12.67
$C_{19}H_{38}O_2$ (298).   "   " 76.51, " 12.75
              Found.   " 76.15, " 12.80

The silver salt was prepared by precipitating a dilute alcoholic solution of the neutral potassium soap with silver nitrate. The white amorphous precipitate was filtered off and washed thoroughly with dilute alcohol and with 95 per cent alcohol. The silver salt was insoluble in water and was apparently insoluble in hot alcohol or in warm ether. It dissolved in hot benzene or toluene, and, on cooling, these solutions set to solid perfectly transparent gels.

The dried preparation was ignited at a low heat in a porcelain crucible and the residue of metallic silver weighed.

Analysis—0.2148 gm. substance: 0.0573 gm. Ag
$C_{18}H_{35}O_2Ag$ (390.88). Calculated. Ag 27.60
$C_{19}H_{37}O_2Ag$ (404.88).   "   " 26.64
              Found.   " 26.67

The values found for the molecular weight of this acid, both by titration and by the silver salt, agree better with the calculated value for a C_{19} acid than with that of a C_{18} acid. It is not possible from the present data to determine the actual composition of the acid and it is not impossible that it may be a mixture of two higher acids since odd carbon acids are rarely, if ever, found in nature. It is very evident, nevertheless, that this liquid saturated fatty acid is very similar too, if not identical with, the tuberculostearic acid (9) which we isolated from the acetone-soluble fat of the human tubercle bacillus.

Wax Fraction

As has been mentioned earlier, 2.0 gm. of a wax-like material separated on cooling the original soap solution. The dry substance was suspended in ether, but it did not dissolve. On passing dry hydrochloric acid into the mixture, the substance, which was evidently a potassium salt, dissolved, while some potassium chloride separated. The ethereal solution was washed with water, dried with sodium sulfate, filtered, and the ether was then evaporated, yielding 1.9 gm. of a waxy solid. The substance was twice precipitated from ethereal solution by adding acetone and cooling when 1.54 gm. of a nearly white powder were obtained. The powder melted at 62–63°. For further purification, the substance was dissolved in 100 cc. of ether, treated twice with norit, filtered, and washed with ether. The colorless solution was concentrated to 50 cc., an equal volume of methyl alcohol added, and the mixture cooled. A snow-white powder consisting of fine globular particles separated, and, after drying *in vacuo*, weighed 1.4 gm.

The substance could not be obtained in crystalline form. It was readily soluble in warm ether and moderately soluble in hot acetone, but from these solutions there separated on cooling only colorless globular particles. Similar results were obtained with various mixed solvents. It was very slightly soluble in hot methyl or ethyl alcohol, and when these solutions were cooled only white amorphous flakes separated.

The purified substance melted at 62–63°, solidified at 56–55°, and remelted at 62°. In the Liebermann-Burchard reaction it gave no coloration whatever. It was evidently an unsaturated compound because in chloroform solution it decolorized a solution of bromine. The substance was optically active and in chloroform solution the specific optical rotation was +4.2°. It acted as an acid and evidently had a high molecular weight.

Titration—0.2457 gm. of substance dissolved in 125 cc. of absolute alcohol and 50 cc. of ether, with phenolphthalein as indicator, required 2.17 cc. of 0.1 N alcoholic KOH. Found: mol. wt., 1130.

Microanalysis—Found, C 80.98, 81.04, H 13.17, 13.22

In solubility and appearance this substance resembles the so called unsaponifiable wax obtained from the human tubercle bacillus, but

it possesses important differences in properties from the latter compound in that it is unsaturated and is optically active. We hope to find larger quantities of this interesting compound in some of the other lipid fractions from the leprosy bacillus so that we may be able to investigate its composition more closely.

Polysaccharide Fraction

The alcohol-insoluble residue which remained after saponifying the phosphatide was dissolved in 25 cc. of water. The solution, which was alkaline in reaction, was acidified with acetic acid, treated with norit, and filtered, when a perfectly clear and nearly colorless filtrate was obtained. The norit was washed thoroughly with water, the filtrate and washings being combined and diluted to about 200 cc. with water.

Precipitation with Lead Acetate

The solution was mixed with 20 per cent lead acetate until no further precipitation was evident. On standing, the mixture gelatinized, but it liquefied on warming. On cooling, the insoluble lead salt settled, leaving a clear supernatant solution which was not gelatinous. The precipitate was filtered off on a Buchner funnel and washed thoroughly with cold water. To the clear filtrate were added a few cc. of a lead acetate solution which did not cause any immediate precipitate, but after standing for a few minutes the solution gelatinized forming a transparent solid jelly which after a short while turned opaque. The mixture liquefied on warming and a slight amount of an insoluble precipitate separated. The precipitate was filtered off, washed with water, and combined with the insoluble lead salt obtained at first. The filtrate was examined for polysaccharide as will be described later.

Lead Acetate Precipitate. Isolation of Barium Glycerophosphate

The lead precipitates were suspended in water and decomposed with hydrogen sulfide. After the lead sulfide had been filtered off and washed with water, the filtrate was concentrated under reduced pressure and finally dried in a vacuum desiccator until a thick syrup remained. The syrup, which weighed 1.7 gm., was stirred up with about 100 cc. of 95 per cent alcohol. A slight amount of an amorphous white precipitate which was insoluble in the alcohol

was filtered off, washed with alcohol, and dried *in vacuo*.   This alcohol-insoluble substance, which weighed 0.2 gm., was combined with the polysaccharide fraction.

The alcoholic solution, which was strongly acid in reaction, was neutralized with barium hydroxide, when a white precipitate separated.   The precipitate was filtered off on a Buchner funnel, washed with alcohol, and dried.   The substance, which weighed 2.5 gm., was treated with 20 cc. of water, when an insoluble portion remained which was filtered off, washed with water, and dried. The water-insoluble substance weighed 1 gm. and, as it gave in nitric acid solution an immediate yellow precipitate with ammonium molybdate, it probably consisted of inorganic barium phosphate.

The filtrate from the insoluble barium salt was mixed with an equal volume of alcohol, when a voluminous white amorphous precipitate separated.   After this precipitate had been filtered off, washed with alcohol, and dried, it was redissolved in water and a trace of insoluble matter filtered off.   The clear filtrate was precipitated by adding an equal volume of alcohol.   The precipitate was collected on a Buchner funnel, washed with alcohol, and dried. It formed a snow-white amorphous powder and weighed 1.5 gm.

Microanalysis—Found, P 9.16, 9.35, Ba 40.40, 40.50; calculated for $C_3H_7O_6PBa + 2H_2O$, P 9.02, Ba 40.01

While the properties of this barium salt agreed with those of barium glycerophosphate and the analytical results for phosphorus and barium are in close agreement with the calculated values, yet the substance was not a pure barium glycerophosphate because the percentage of hydrogen found was too low and the percentage of carbon was nearly 2 per cent too high.

Isolation of Polysaccharide

The filtrate from the lead acetate precipitate was freed from excess of lead with hydrogen sulfide, filtered, concentrated under reduced pressure to a syrup, and the latter dried in a vacuum desiccator.   The syrup was dehydrated by grinding under absolute alcohol in a mortar until a snow-white powder was formed. The powder was filtered off on a Buchner funnel, washed with absolute alcohol, and dried in a vacuum desiccator.   The sub-

stance, which weighed 8.1 gm., was united with the 0.2 gm. of the alcohol-insoluble precipitate mentioned in the preceding section, thus giving 8.3 gm. of polysaccharide. It was dissolved in 20 cc. of water, 5 cc. of glacial acetic acid were added, and the solution was poured into 400 cc. of 95 per cent alcohol with constant stirring. The white amorphous precipitate which separated was filtered off, washed with alcohol, and dried. The substance was dissolved in 10 cc. of water, the solution was filtered, and the flask and filter were washed with 10 cc. of water. The clear colorless filtrate was diluted with 20 cc. of glacial acetic acid and the mixture poured with constant stirring into 500 cc. of absolute alcohol. A fine white flocculant precipitate separated and settled rapidly. The precipitate was filtered off, washed with absolute alcohol, and dried *in vacuo*. It formed a snow-white powder and weighed 5.8 gm. The mother liquors were concentrated and treated with absolute alcohol, when 1.1 gm. of a snow-white amorphous powder were obtained.

The purified polysaccharide contained organic phosphorus and showed an acid reaction on moist litmus paper. It gave no reduction when boiled with Fehling's solution, but, after the aqueous solution had been boiled for several minutes with dilute acid and then neutralized, it reduced Fehling's solution. The substance had no melting point. Heated in a capillary tube, it began to swell at 115° and to turn yellow at 250°, but it had not melted at the boiling point of sulfuric acid.

Microanalysis—Found, P 4.70, 4.71, ash 20.36, 20.76, C 33.35, 33.32, H 5.36, 5.30 per cent

Hydrolysis of Polysaccharide

The purified polysaccharide, 2 gm., was refluxed with 250 cc. of 3.5 per cent sulfuric acid until the reducing power of the solution, as determined by the Shaffer-Hartmann (12) method, was constant. The maximum reduction was attained after 3 hours and there was no further change after the solution had been refluxed for 7 hours.

The faintly straw-colored solution was made slightly alkaline with barium hydroxide after which carbon dioxide was bubbled through until the reaction was neutral. The precipitate was

filtered off and washed thoroughly with hot water. Since the precipitate was found to contain some barium phosphate in addition to barium sulfate, it is evident that phosphoric acid had been formed from an organic phosphorus compound during the hydrolysis.

The filtrate from the insoluble barium salts was concentrated under reduced pressure to a volume of 50 cc. and the solution mixed with an equal volume of alcohol. A trace of an insoluble precipitate was filtered off and washed with dilute alcohol. The slight amount of precipitate obtained indicated that only traces of an organic phosphoric acid such as glycerophosphoric acid was present. The slight excess of barium contained in the solution was removed quantitatively with sulfuric acid.

Precipitation of Mannose Phenylhydrazone

The solution, after the barium sulfate had been filtered off, was concentrated under reduced pressure to a volume of about 20 cc. and mixed with 2 gm. of freshly distilled phenylhydrazine dissolved in 3 cc. of alcohol. Mannose phenylhydrazone began to crystallize almost immediately, and after the mixture had stood overnight the crystals were filtered off on a Buchner funnel and washed with water and with alcohol. The product, after it had been dried *in vacuo*, formed a light yellowish crystalline powder which weighed 1.0 gm., corresponding to 0.66 gm. or 33 per cent of mannose. The substance was recrystallized from hot 60 per cent alcohol yielding 0.65 gm. of nearly colorless characteristic massive plate-shaped crystals. The powdered crystals melted at 195–196° and there was no depression of the melting point when the substance was mixed with pure mannose phenylhydrazone.

Isolation of Inosite

The excess of phenylhydrazine in the filtrate from the mannose phenylhydrazone was removed with benzaldehyde, the hydrazone was filtered off, and the filtrate, after it had been extracted repeatedly with ether, was concentrated to a syrup. On scratching, crystallization began, and after a few hours the mixture was filled solidly with crystals. The latter were stirred up with cold 70 per cent alcohol, filtered, and washed with 70 per cent alcohol, and finally washed with alcohol and ether. The filtrate and wash-

ings on standing deposited a small amount of needle-shaped crystals which were filtered off and combined with the crude inosite.

The filtrate was evaporated to dryness, when 0.3 gm. of a syrup remained which was examined, as will be described later.

The crude inosite was recrystallized four times from water by the addition of alcohol and was obtained in colorless prismatic needles which weighed 0.234 gm. From the mother liquors we obtained an additional 0.054 gm. of needle-shaped crystals. The total amount of inosite obtained was therefore 0.288 gm., corresponding to 14.4 per cent of the polysaccharide. The crystals gave the reaction of Scherer and melted at 224–225°. There was no depression of the melting point when some of the substance was mixed with inactive inosite prepared from phytin.

Examination of Residual Syrup.   Preparation of Glucosazone

The syrup obtained on the evaporation of the filtrate from the inosite crystals gave a heavy reduction when some of it was boiled with Fehling's solution. It gave no pentose color reactions, but when heated with 12 per cent hydrochloric acid and resorcinol gave a bright red color characteristic of ketoses.

The syrup was dissolved in 6 cc. of water and the solution mixed with phenylhydrazine hydrochloride and sodium acetate. The solution was allowed to stand for some time at room temperature, but no precipitate formed, thus showing that no mannose was present. The solution was then heated in a boiling water bath and typical glucosazone crystals began to separate at the end of 5 minutes. After heating for 1½ hours, the mixture was cooled and the crystals filtered off, washed with water, and dried *in vacuo.* The product, which weighed 0.11 gm., was dissolved in 15 cc. of alcohol, 15 cc. of warm water were added, the solution was treated with norit, and filtered. As the solution cooled, fine yellow needle-shaped crystals separated and these were filtered off, washed with alcohol, and dried *in vacuo.* The powdered crystals melted with decomposition at 208° and there was no depression of the melting point when some of the substance was mixed with pure glucosazone.

The formation of glucosazone would indicate that the syrup contained a hexose sugar, and, since the ketose color reaction was

positive, it seems probable that the sugar was either fructose or a mixture similar to invert sugar.

It is evident from the data which we have obtained that the polysaccharide from the leprosy bacillus phosphatide, when boiled with dilute sulfuric acid, is completely hydrolyzed in 3 hours. The hydrolysis products which are formed consist of phosphoric acid, 33 per cent of mannose, 14.4 per cent of inosite, and about 15 per cent of some hexose sugar.   These values are minimal since losses in the various operations are inevitable.

TABLE I

Cleavage Products from Phosphatide of Bacillus lepræ

	First analysis		Second analysis	
	gm.	*per cent*	*gm.*	*per cent*
Crude fatty acids.........................	8.68	57.8	13.7	54.8
" wax and pigment..................	1.49	9.9	2.8	11.2
Total ether-soluble constituents...........	10.17	67.8	16.5	66.0
Solid saturated fatty acids, mainly palmitic acid...................................	2.79	18.6	6.2	24.8
Liquid fatty acids........................	4.47	29.8	7.3	29.2
Reduced acids, palmitic and stearic acids...	2.07	13.8	4.4	17.6
Liquid saturated fatty acid, analogous to tuberculostearic acid....................	2.03	13.5	2.7	10.8
Crude polysaccharide.....................			8.3	33.2
" barium glycerophosphate..........			1.5	6.0

In conclusion we give in Table I a summary of the cleavage products which have been isolated from the leprosy bacillus phosphatide.

SUMMARY

1. The phosphatide isolated from *Bacillus lepræ* has been analyzed and found to be similar in composition to the phospha-

tides isolated from other acid-fast bacteria. Certain differences are, however, evident: (a) The phosphatide is exceedingly stable and cannot be hydrolyzed completely with dilute aqueous acid. (b) The solid saturated fatty acid is not homogeneous. It consists principally of palmitic acid with a slight admixture of a new fatty acid of high molecular weight which could not be identified. (c) Two unsaturated fatty acids are present which on catalytic reduction are converted into palmitic acid and stearic acid.

2. In addition to fatty acids, the ether-soluble constituents also contain a small amount of a wax-like substance. This compound is unsaturated, optically active, and of high molecular weight.

3. The liquid saturated fatty acid is optically inactive and is similar to or identical with tuberculostearic acid.

4. When the phosphatide is saponified with dilute alcoholic potassium hydroxide, only the fatty acids and glycerophosphoric acid are split off, while the polysaccharide complex is left intact.

5. The polysaccharide, when hydrolyzed with dilute acid, yields approximately 2 parts of mannose, 1 part of inosite, and 1 part of a reducing hexose which is probably invert sugar or fructose.

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THE CHEMISTRY OF THE LIPIDS OF TUBERCLE BACILLI

XXVIII. STUDIES ON PHTHIOIC ACID. ISOLATION OF A LEVORO-
TATORY ACID FROM THE PHTHIOIC ACID FRACTION OF
THE HUMAN TUBERCLE BACILLUS*

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INTRODUCTION

It has been shown in earlier investigations in this laboratory that all of the lipid fractions isolated from the human tubercle bacillus contain optically active higher saturated fatty acids which are liquid or very low melting solids at ordinary temperature (1, 2). The lead salts of these acids are soluble in ether. The crude liquid saturated fatty acids as obtained from the various lipid fractions show considerable variation in physical properties. The phosphatide yields an acid which is a mobile oil, and its specific rotation is +1.60°. The corresponding acid from the purified wax is a thick oil solidifying at 18° with a specific rotation of −1.65°. From the so called *soft wax*, an acid was isolated which melted at 22.5°, and its rotation was +4.85°. The acetone-soluble fat (3) contained a larger amount of the liquid saturated fatty acids; and in this case the methyl esters were prepared and fractionated in a high vacuum (4). In this manner the esters were separated into two principal fractions that differed in boiling point by about 50°. The low boiling fraction of the ester was optically inactive and, on saponification, gave a liquid saturated acid which was found to be isomeric with stearic acid, for which reason it was designated as *tuberculostearic acid*. The higher boiling ester yielded an acid which melted at 28°, and its specific rotation was +7.98°.

It has been established through the investigations of Sabin and collaborators (5) that the dextrorotatory acid, when injected intraperitoneally, stimulates the proliferation of monocytes, epithelioid cells, and giant cells, with the formation of tubercular tissue.   In order to indicate the relation of this acid to the formation of tubercles in tuberculosis, it was named *phthioic acid.*

In view of the peculiar and interesting physical and biological properties of phthioic acid, we have been anxious to obtain some information concerning its structure, but, before any experiments in this direction could be undertaken, it was necessary to secure a larger amount of the acid.   The object of the present investigation was to prepare material which could be used in studies of the structure of phthioic acid.   For this purpose, relatively large amounts of crude lipid fractions obtained from the human tubercle bacillus, Strain H-37, were collected during the past several years. In working up this material, our main interest centered on the optically active fraction of the liquid saturated fatty acids; other fractions being set aside for some future use.

The opinion was expressed in the earlier publication (4) that the liquid saturated fatty acids obtained from the human tubercle bacillus, Strain H-37, probably contained other new fatty acids in addition to tuberculostearic acid and phthioic acid.   This prediction has now been verified in the isolation of a levorotatory fatty acid of high molecular weight from the phthioic acid fraction.

It was observed during the fractionation of the higher boiling portion of the methyl esters, which contained the optically active material, that the first fractions had the highest dextrorotation, while, as the boiling point rose, the dextrorotation declined. Continued fractionation of both the top and bottom fractions finally resulted in a separation into dextro- and levorotatory esters. The separation was extremely slow, difficult, and laborious, and could not be carried to completion by fractional distillation of the esters.   The fractional precipitation of the esters from acetone solution afforded a more rapid separation, but it was probably not completely satisfactory.   The purification of the levorotatory ester was accomplished in this manner.

The purified dextrorotatory phthioic acid isolated in this investigation possessed the same composition and gave the same biological reactions as have been determined and described previously.   The

analytical results were in agreement with the composition of a hexacosanic acid, $C_{26}H_{52}O_2$. The specific rotation was $+11.96°$, which is about 4° higher than that formerly observed, while the melting point was lower, the purified acid being a liquid at room temperature.

The levorotatory acid has probably not yet been obtained in absolutely pure form. The rotation of the purest specimen was $-6.14°$, the melting point was 48–50°, and the composition agreed approximately with the formula $C_{30}H_{60}O_2$.

EXPERIMENTAL

For the present work we employed numerous fractions of crude acetone-soluble fat, as well as mixed fatty acids which had been collected during the past 3 or 4 years in experiments with the human tubercle bacillus, Strain H-37. Some of the fat fractions had been prepared in the Mulford Biological Laboratories, but a portion of the material had been isolated in this laboratory. The fat was saponified with alcoholic potassium hydroxide, the unsaponifiable matter was removed by extraction with ether, and the fatty acids were isolated and separated by means of the lead soap-ether method into solid and liquid fatty acids.

The liquid fatty acids obtained in this manner consist, as shown in earlier investigations (3), of a mixture of unsaturated acids and liquid saturated fatty acids. In order to separate such a mixture of liquid unsaturated and liquid saturated fatty acids, we have developed a method which depends upon the catalytic hydrogenation of the unsaturated fatty acids, followed by the removal of the reduced acid by means of the lead soap-ether treatment. The method is cumbersome, slow, and inconvenient, but so far it is the only way by which the specific liquid saturated fatty acids, characteristic of the tubercle bacillus and other acid-fast bacteria, can be isolated. The crude liquid fatty acids obtained from the tubercle bacillus fat contain, unfortunately, some substance which poisons the catalyst. It is, therefore, impossible to hydrogenate the crude acids directly. It is necessary first of all to esterify the crude liquid fatty acids and to distil and redistil the esters. Secondly, the purified esters are saponified, the free acids are isolated and are subjected to hydrogenation, which now proceeds smoothly and rapidly to completion. The various operations

involved in the purification processes occasion considerable, but unavoidable, losses of material.

The crude fat, which weighed 360.5 gm., yielded 50.2 gm. of unsaponifiable matter, 288 gm. of fatty acids, and 19.4 gm. of water-soluble constituents. The unsaponifiable matter and the water-soluble fraction were reserved for future investigations.

The crude fatty acids were combined with similar fatty acids which had been obtained in analyses of other lipid fractions from the human tubercle bacillus, giving a total of 404.9 gm. of mixed fatty acids. The mixture, after separation by the lead soap-ether method, gave 284 gm. of liquid fatty acids and 107 gm. of solid acids. The solid fatty acids were not investigated at this time, but were saved for future use.

The liquid acids were converted into methyl esters and the latter were distilled in a high vacuum. The distillate which came over between 115–260° at a pressure of about 0.001 mm. weighed 230 gm. The thick viscous non-volatile residue weighed 53 gm. and was not further investigated. After the ester had been redistilled, it was saponified. The saponification mixture was extracted with ether and the ethereal extract evaporated to dryness, when 4.6 gm. of a soft semisolid material remained and was discarded. The fatty acids were isolated and reduced in alcoholic solution with hydrogen and platinum oxide (6). The reduction product was separated by repeating the lead soap-ether treatment, and we obtained 56 gm. of reduced solid acid which was reserved, for future investigation. The ether-soluble lead soaps yielded 160 gm. of liquid saturated fatty acids. The acid was a faintly yellowish oil. The material was saturated, as shown by the fact that it did not absorb any iodine when tested by the Hanus method.

Fractionation of Methyl Esters of Liquid Saturated Fatty Acids

The liquid saturated fatty acids were combined with 26.9 gm. of similar acids which had been isolated in the analysis of the so called *soft wax* (2). The mixed acids, 186.9 gm., were converted into methyl esters. The ethereal solution of the esters was washed repeatedly with dilute potassium hydroxide and with water. After the ethereal solution had been dried with sodium sulfate, the ether was distilled off. The esters, weighing about 192 gm., formed a faintly yellowish mobile oil.

A preliminary separation was made into low boiling and high boiling fractions of the esters. It is known from our earlier investigation (4) that the mixed esters contain a relatively large proportion of the low boiling methyl ester of tuberculostearic acid, and this fraction was removed first. At a pressure of about 0.003 mm. the first fraction was distilled off with the temperature of the bath at 150–160°. The distillate was a mobile, faintly yellowish oil which weighed 65 gm. The ester was optically inactive and its other properties corresponded to those described for the methyl ester of tuberculostearic acid. This material was reserved for future investigation.

For the fractionation of the higher boiling esters, an all-glass apparatus was used of similar construction to the one which we have previously described (4). In the present case, however, a trap, cooled in liquid air, was introduced between the condenser and the mercury diffusion pump, and this arrangement served to improve the vacuum very greatly. As measured on a MacLeod gage, the pressure was estimated to be less than 0.0001 mm., and this high vacuum could be maintained indefinitely without showing any change during the distillations. The fractionating column of the apparatus was wound with a nichrome band which could be heated to any desired temperature by an electric current. A further improvement in the apparatus was a side arm fused into the fractionating flask through which the flask could be filled or emptied without cutting down the whole apparatus. After the flask had been filled, the side arm was fused off, while at the end of the distillation the side arm was opened for refilling or for cleaning the apparatus.

The higher boiling esters distilled at a rapid rate when the temperature of the bath was kept at 200°, but everything distilled over at this temperature, hence there was no fractionation, and when the temperature of the bath was much below 200° there was practically no distillation. By conducting the fractionations very slowly, and with the fractionating column heated to the same temperature as the bath, it was possible to effect a separation of the mixture. Since the higher boiling esters are optically active, the fractionation could be followed by observing the optical rotation of the various fractions.

The fractionations were conducted at the lowest possible tem-

perature, beginning at 175° and gradually increasing the tempera-
ture, fractions being collected at 5° intervals. The ester vaporized
very slowly, a drop coming over every 2 or 3 minutes. There was
no sharp break in the vaporization, but the highest dextrorotation
was always obtained in the first fractions. The fractions having
approximately the same rotation were united and refractionated
many times in the manner mentioned above. We finally obtained
a top fraction which had a specific optical rotation of about $+11.0°$,
while intermediate fractions varied in rotation from $+11.0$ to $+0.8°$.
It was also observed that the less volatile portions of the ester had
the lowest dextrorotation, and, when a sufficient amount of this
material had been accumulated, it was further fractionated in the
same manner, but at slightly higher temperatures. The final
highest boiling fraction came over at about 220°, and the rotation
was $-1.0°$. These upper and lower limits in the specific rotation
could not be changed by further distillations. The results so
far obtained indicated clearly that the original mixture of esters
consisted of an optically inactive portion, such as the methyl
ester of tuberculostearic acid, the dextrorotatory phthioic acid, and
a third, heretofore unknown ester which was levorotatory.

It is worthy of note that the liquid air trap never contained a
trace of esters, but always contained, on the side nearest to the
mercury diffusion pump, a distinctly visible layer of finely divided
mercury. The esters must therefore have an extremely low vapor
pressure.

The ester fractions which possessed a low dextrorotation solidi-
fied partly when allowed to stand for several hours at a temperature
slightly below 20°, while the ester with the highest dextrorotation
remained liquid under the same conditions. The material did not
crystallize in the ordinary sense of this term, but the solid particles
consisted of colorless, very long, round, thread-like forms with
round dots distributed at even intervals.

It occurred to us, therefore, that a better separation of the
dextro and levo forms might be accomplished by cooling the esters
and filtering off the solid portions. An experiment was tried in
which 12.2 gm. of the ester with $[\alpha]_D^{20} = +0.8°$ was cooled in the
ice box for 1 hour. The solid which had separated was filtered
off on a Buchner funnel and freed as far as possible from the ad-
hering liquid ester. The solid portion which remained in the

funnel weighed 5 gm. and its specific optical rotation was $-2.2°$. In another experiment some of the same ester fraction was dissolved in a little acetone and the solution was cooled in ice water. A small amount of white solid substance separated and was filtered off and washed with ice-cold acetone. The specific rotation of the solid material was $-5.6°$. Various other solvents were tried, but it was found that acetone was the most suitable solvent to use.

Similar experiments were carried out with the ester which had a dextrorotation of $+11°$, but in no case was any increase in rotation obtainable.

Purification of Levorotatory Ester by Precipitation from Acetone

The ester previously mentioned, which had been obtained by fractional distillation, and which had a rotation of $-1.0°$, weighed 8.1 gm. The substance was dissolved in 25 cc. of acetone and the solution cooled in ice water. The solid which separated was filtered off on a Buchner funnel. It was redissolved in 25 cc. of acetone and reprecipitated by cooling. These operations were repeated four times. The solvent adhering to the solid was removed by warming and a current of carbon dioxide. The substance was finally dried in vacuo. The substance formed a white crystalline solid at room temperature and weighed 4.3 gm.

Rotation—0.7664 gm. of substance was dissolved in ether and made up to 10 cc. In a 1 dm. tube the rotation was $-0.495°$.

$$[\alpha]_D^{22} = \frac{-0.495 \times 100}{7.664 \times 1} = -6.45°.$$

Other intermediate fractions of the distillate were treated in the same manner in acetone solution and small amounts of solid esters were obtained. The rotation of these fractions was also approximately $-6.0°$.

The fractionations from acetone could only be carried out in a cold room since otherwise the material dissolved before it could be filtered off. Since it appeared impossible to carry the purification of the levorotatory fraction any further, the substance was analyzed.

Analysis—0.1134 gm. substance: 0.1365 gm. H_2O and 0.3299 gm. CO_2
$C_{31}H_{62}O_2$ (466). Calculated. C 79.82, H 13.30
Found. " 79.34, " 13.34

Preparation of Free Acid

The various fractions of the ester with a levorotation of about 6.0° were combined, total weight 5.4 gm., and saponified with alcoholic potassium hydroxide. The free acid, after having been isolated and dried *in vacuo*, weighed 5.3 gm. and formed a white semicrystalline solid. The substance had no sharp melting point. When heated, it liquefied gradually to a clear oil at 48–50°.

Rotation—0.5160 gm. of substance dissolved in absolute alcohol and made up to 10 cc. gave in a 1 dm. tube a reading of $-0.317°$; hence, $[\alpha]_D^{20} = -6.14°$.

Much time was consumed in efforts to purify the acid by precipitation from various solvents, fractionation of the potassium salt, and especially in attempts to prepare salts with active alkaloids, but all efforts in this direction were futile since no increase in the levorotation could be obtained. The acid was therefore analyzed and its composition determined.

Analysis—0.1093 gm. substance: 0.1305 gm. H_2O and 0.3191 gm. CO_2
$C_{30}H_{60}O_2$ (452). Calculated. C 79.64, H 13.27
Found. " 79.62, " 13.36

Titration—0.5272 gm. of substance dissolved in neutral alcohol, with phenolphthalein as indicator, required 11.31 cc. of 0.1 N alcoholic KOH.

$C_{30}H_{60}O_2$. Mol. wt. calculated, 452; found, 466

The silver salt was prepared by adding a solution of silver nitrate to the neutral, titrated solution of the potassium salt of the acid. The white precipitate which separated was filtered off, washed with dilute alcohol, and with alcohol. After the substance had been dried in a vacuum desiccator, it formed a faintly pink-colored amorphous powder. The silver salt appeared to be insoluble in ether, but dissolved completely in warm benzene, and from this solution it was precipitated by adding methyl alcohol in the form of white amorphous flakes. Heated in a capillary tube, the salt began to sinter at 80–85°, gradually turned brown in color, and melted to a brown liquid at 175–180°. For analysis the substance was dried at 105° *in vacuo*, ignited at a low heat in a porcelain crucible, and the residue of metallic silver weighed.

0.2277 gm. substance: 0.0434 gm. Ag

$C_{30}H_{59}O_2Ag$ (558.88). Calculated, Ag 19.30; found, Ag 19.06

It is evident from the analytical results that the levorotatory acid differs in composition from phthioic acid. The values obtained agree approximately with the calculated composition of a saturated C_{30} acid. It must be stated, however, that we have in this case no adequate criteria as to the purity of the acid, and the chances are that it is not homogeneous. Since the substance is optically active and since its properties, such as solubility and melting point, differ entirely from those of ordinary straight chain acids, it is evident that the structural configuration of this acid must be represented by a branching chain.

The biological reactions of the levorotatory acid, as determined by Dr. Sabin and her collaborators, have been found to be distinctly different from those of phthioic acid. The present acid appears to act merely as an irritant, without causing the tubercular tissue reaction which is typical of phthioic acid.

Dextrorotatory Phthioic Acid

The top fraction of the ester, corresponding to the methyl ester of phthioic acid, had a specific rotation of about $+11.0°$ and weighed 25.8 gm. It was a thick, faintly yellow oil at room temperature. On analysis, the following values were obtained.

0.1246 gm. substance: 0.1452 gm. H_2O and 0.3600 gm. CO_2

$C_{27}H_{54}O_2$ (410). Calculated. C 79.02, H 13.17

Found. " 78.79, " 13.04

The ester was saponified with 5 per cent alcoholic potassium hydroxide; the soap solution was diluted with water and extracted with ether. When the ethereal solution was evaporated to dryness, there was hardly any noticeable residue. The soap solution was acidified with hydrochloric acid and the acid which separated was extracted with ether. The ethereal solution was washed with water until the washings were free from hydrochloric acid, after which it was treated with norit and filtered. The solution, which was practically colorless, was dried with sodium sulfate, filtered, and the ether distilled. The residue, after having been dried *in vacuo*,

formed a thick, faintly yellowish oil which remained liquid at ordinary room temperature.

Rotation—0.6510 gm. of substance dissolved in absolute alcohol and made up to 10 cc. gave in a 1 dm. tube a reading of $+0.678°$; hence, $[\alpha]_D^{21} = +10.41°$.

Analysis—0.1151 gm. substance: 0.1347 gm. H_2O and 0.3314 gm. CO_2
$C_{26}H_{52}O_2$ (396).   Calculated.   C 78.78, H 13.13
Found.   " 78.52, " 13.09

Titration—0.4297 gm., 0.5580 gm. of substance dissolved in neutral alcohol, with phenolphthalein as indicator, required 10.57 cc. and 13.86 cc. of 0.1 N alcoholic KOH.

$C_{26}H_{52}O_2$ (396).   Mol. wt. calculated, 396; found, 406, 402

The silver salt was prepared by adding a solution of silver nitrate to the neutral solution of the potassium salt of the acid.   The white precipitate was filtered off, washed with dilute alcohol and with alcohol, and dried *in vacuo*.   The dried silver salt was soluble in warm benzene, but it was precipitated from this solution by methyl alcohol as a white, curdy mass.   The supernatant solution was decanted and the residue treated with ether when the precipitate dissolved.

For analysis the silver salt was ignited at a low heat in a porcelain crucible and the metallic silver weighed.

0.2936 gm., 0.2498 gm. substance: 0.0625 gm., 0.0535 gm. Ag
$C_{26}H_{51}O_2Ag$ (502.88).   Calculated.   Ag 21.45
Found.   " 21.28, 21.41

Various experiments were tried to purify this acid by precipitation from acetone and other solvents on cooling in a freezing mixture, but in no case could we observe any change in the rotation or in the composition of different fractions.   An attempt was made to fractionate the potassium salt of the acid from 50 per cent alcohol.   The acid recovered from the salt which separated on cooling the solution had practically the same rotation and composition as the starting material.   The acid which was recovered from the soluble portion of the potassium salt was converted into the methyl ester and the latter was again fractionally distilled at a

pressure of 0.0001 mm. in the manner already described. The main portion of the ester, which had a rotation of +11.42°, was saponified and the free acid isolated. The acid thus purified was a liquid at room temperature, but solidified on cooling in ice water.

Rotation—0.7441 gm. of acid dissolved in alcohol and made up to 10 cc. gave in a 1 dm. tube a reading of +0.89°; hence, $[\alpha]_D^{20}$ = +11.96°.

Titration—0.4259 gm. of acid dissolved in neutral alcohol, with phenolphthalein as indicator, required 10.46 cc. of 0.1 N alcoholic KOH. Mol. wt. found, 407.

Analysis—0.1066 gm. substance: 0.1207 gm. H_2O and 0.3057 gm. CO_2
Found. C 78.21, H 12.67

There are no significant differences in composition, so far as one can tell by analyses, between the purified phthioic acid here described and the specimen which was first isolated from the acetone-soluble fat. The purified acid has, however, a higher dextrorotation and a lower melting point, and these changes in properties are no doubt due to the removal of the levorotatory acid.

SUMMARY

A comparatively large quantity of the liquid saturated fatty acids from mixed lipids of the human tubercle bacillus has been examined.

In addition to tuberculostearic acid and phthioic acid, a new acid belonging to this series has been isolated. This new acid is levorotatory, $[\alpha]_D^{20}$ = −6.14°. It is solid at ordinary room temperature and melts at 48–50°. It appears to be a saturated fatty acid having the composition $C_{30}H_{60}O_2$.

The purified phthioic acid obtained in this investigation is liquid at room temperature, $[\alpha]_D^{20}$ = +11.96°, and in composition it corresponds to a hexacosanic acid, $C_{26}H_{52}O_2$.

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DIRECTIVE INFLUENCES IN BIOLOGICAL SYSTEMS

II. LIPASE ACTIONS OF TYPES I AND II PNEUMOCOCCI

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INTRODUCTION

Different strains of bacteria might be expected to show different and possibly characteristic enzyme actions. The results of the study of such actions, in as far as relations and classifications based upon them are concerned, have been disappointingly meager. It will be shown in this paper that in the study of certain lipase or ester-hydrolyzing actions of pneumococci, the medium in which the bacteria are growing plays an important part, and that in order to determine whether regularities exist, the medium must be kept constant or the differences in its properties noted.

EXPERIMENTAL

Methods

Media—Beef heart broths were used as media. They were prepared as follows (1): 500 gm. of chopped, fat-free beef heart were well mixed with 1 liter of water and allowed to stand overnight in the ice box. The following morning the mixture was slowly heated to boiling and boiled for 5 to 10 minutes until the meat was thoroughly clumped. The meat was then removed by straining through cheese-cloth. The volume of infusion was measured, and 1 gm. of peptone (Fairchild) and 0.5 gm. of sodium chloride were added for each 100 cc. The peptone was dissolved in a small quantity of water and added with stirring. 20 cc. of N NaOH solution per liter were added and the mixture brought to

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a boil. The required amount of N NaOH solution to bring the mixture to pH 7.9 to 8.0, phenol red being used as indicator, as determined by preliminary tests, was added. The broth was then boiled for 10 minutes, filtered through filter paper three times, bottled, and autoclaved for 20 minutes at 15 pounds pressure. The final pH was usually 7.4 to 7.6. 10 to 15 liters of broth, made from three to five beef hearts, were prepared at a time. The beef hearts were obtained fresh from the slaughter-house and chopped within an hour.

Total and formol nitrogen content as well as total and fermentable reducing action was determined for all the broths. No broth was used which showed an unfavorable fermentable reducing action, the required content for a satisfactory broth being 0.5 to 1.2 mg. of reducing substance calculated as glucose per cc. (1).

Bacteria—Although more than 20 types of pneumococci[1] were tested, the results for Types I and II only were repeated a sufficient number of times to warrant inclusion in this report. The strains were either freshly isolated or had been transplanted in (horse) blood broth and mice in order to maintain their virulence until used. The inoculums for the experiments consisted of the supernatant liquid from an 18 hour blood broth culture of the virulent culture from a mouse heart. To 300 to 350 cc. of the medium 5 to 10 cc. of the inoculum were added. If growth was scanty after 24 hours, another 5 cc. were added. After 24 or 48 hours abundant growth 20 to 30 cc. of toluene were added, the mixtures shaken twice each day, and allowed to stand at room temperature for 15 to 20 days. For the enzyme studies, the mixtures were diluted with equal volumes of water, brought to pH 7.0, and 15 cc. portions used for the tests.

Enzyme Tests—Ester-hydrolyzing actions were determined as described in the preceding paper of this series (2). It will suffice to present results for four esters here, phenyl acetate, glyceryl triacetate, methyl *n*-butyrate, and benzyl acetate. The method of experimenting was the same as before, 3.4 milli-equivalents of the esters, 22 hours incubation at 37°, titration with 0.1 N sodium hydroxide solution with phenolphthalein as indicator, duplicate determinations, and the usual blanks.

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[1] Thanks are due Miss Georgia Cooper for supplying these strains.

Results

If a definite strain of bacteria, such as a given type of pneumo-coccus, is seeded into a broth and after growth and killing of the bacteria the ester-hydrolyzing actions are determined, as a rule definite actions are obtained. If the experiment is repeated with another broth, prepared with similar materials and in the same way as far as possible, quite different enzyme actions may be found. It is therefore necessary, at any rate for a first study of the subject, to compare the actions of different strains in the same broth and then later to compare the actions in different broths.

TABLE I

Ester-Hydrolyzing Actions in Terms of Tenths of Milli-Equivalents of Acid Produced by Broth Media after Growth of Pneumococci

	Type I pneumo-cocci		Type II pneu-mococci		Ratio Col-umns 1 to 3	Ratio Col-umns 2 to 4	Column 5 values PhOAc = 100	Column 6 values PhOAc = 100
	Broth 3 (2 de-termi-nations) (1)	Broth 7 (5 de-termi-nations) (2)	Broth 3 (2 de-termi-nations) (3)	Broth 7 (5 de-termi-nations) (4)	(5)	(6)	(7)	(8)
PhOAc.....	1.27	1.17	0.85	1.15	1.49	1.02	100	100
Gl(OAc)₃...	1.13	1.25	0.74	1.23	1.53	1.02	103	100
MeOCOPr..	1.78	1.13	0.70	0.57	2.54	1.98	170	194
PhCH₂OAc.	0.22	0.18	0.18	0.21	1.2	0.9	80	90

The averages of the results obtained with pneumococcus Types I and II grown in Broths 3 and 7 are presented in Table I.

The hydrolyzing actions on the four esters by Broths 3 and 7 after growth of pneumococcus Type I as shown in Columns 1 and 2 of Table I differ. The differences are especially large for methyl butyrate. The results of similar experiments on these esters with pneumococcus Type II differ much more widely with the two broths (Columns 3 and 4). If the ratios of the actions on each ester of the two types of pneumococci in each broth are taken, and these ratios (Columns 5 and 6) are reduced to the common basis of phenyl acetate = 100 as shown in Columns 7 and 8, the values for the remaining three esters are much the same. That is to say, the factors which increase the actions in a given

broth with one type of pneumococcus increase the actions on the other type as well.

DISCUSSION

The ester-hydrolyzing actions used in this work may be looked upon as sensitive chemical reactions of the materials in question. For the present it is impracticable to ascribe a more definite significance to them. The relation of these enzyme actions to the growth properties of the media and the possible dependence of the different types of pneumococci upon these is unknown.

With these enzyme actions, therefore, as sensitive chemical reagents, the experimental work described leads to the following relations:

Media tested after growth of Type I pneumococcus showed different amounts of hydrolysis upon the four esters tested. Two broth media prepared in the same way, after similar treatment with Type I pneumococci gave different actions on these esters.

Different results were also obtained with Type II pneumococci in the two broths, differing as well from those obtained with Type I.

In other words, uniform enzyme actions, in the sense used with ester-hydrolyzing actions, were not obtained with a definite type of pneumococcus with different lots of broth.

However, after growth of Type I and of Type II pneumococci, the ratios of the actions on the four esters in a given broth had certain values which bear the same relation to each other as the ratios of analogous actions in another broth (Table I, Columns 7 and 8). That is to say, factors which in a given broth resulted in larger actions on methyl butyrate with Type I pneumococcus than with Type II, with another broth might result in a smaller absolute action with Type I, but then would result in a still smaller action with Type II.

SUMMARY AND CONCLUSIONS

In the preparation of broth media for pneumococcus, the ordinary simple chemical tests are limited in so far as a knowledge of their possible influences in biological reactions are concerned. Based upon the study of simple enzyme actions in media containing growth and autolytic products of pneumococci, differences were

found in these broth media prepared and used apparently in the same way.

The experimental evidence presented with two broths and Types I and II pneumococci showed regularities in the enzyme actions only· if each broth is considered individually and the results with one broth not carried over to the other.   If it is permitted to apply the conclusions derived from the study of these enzyme actions to the growth of bacteria themselves, it may be said that results obtained in one medium should not, except in a most general way, be used with another medium.

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THE OXIDATION OF CYSTINE IN ACID SOLUTION*

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Cystine has been regarded as a fairly stable substance when in
acid solution. However, in the present paper there are recorded
the results of experiments, extending over a long period of time,
which indicate a considerable tendency towards atmospheric oxi-
dation when solutions of cystine in hydrochloric acid are exposed
to air for long periods.

The experiments here recorded were begun for the purpose of
following the course of the racemization of cystine in acid solution
at room temperature and at 38° in order to determine the order of
the reaction.

EXPERIMENTAL

A sample of cystine of an ordinary C.P. grade, partially race-
mized, was used. Portions of this sample were dissolved in hydro-
chloric acid of various concentrations and in one sulfuric acid
solution. All solutions contained 1.000 gm. of cystine per 100 cc.
except the most concentrated hydrochloric acid solution (20 per
cent) which because of the decreased solubility of cystine in
this concentration of acid, contained 0.500 gm. per 100 cc. The
solutions were preserved in glass-stoppered bottles but were not
protected from air. They were allowed to stand for a period of
several years, one series at 38° and a duplicate series at room
temperature, and at intervals of several months the optical activity
was measured at 25° with D light. Later, the solutions were
examined to determine the degree and type of decomposition
which had occurred.

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* An abstract of this paper was presented at the meeting of the Federa-
tion of American Societies for Experimental Biology, at Philadelphia, in
April, 1932.

TABLE I

Cha: Rotation Acid So... Cystine at R... Tempera... at Specific R... with D L...

| Time | 0.5 N HCl | | | | 2.5 N HCl | | | | 6 N HCl (20 per cent) | | | | 2.5 N H₂SO₄ | | | |
| | Room temperature | | 38° | | Room temperature | | 38° | | Room temperature | | 38° | | Room temperature | | 38° | |
	$[\alpha]^{25}_D$	$K \times 10^5$	$[\alpha]^{25}_D$	$K \times 10^5$	$[\alpha]^{25}_D$	$K \times 10^5$	$[\alpha]^{25}_D$	$K \times 10^5$	$[\alpha]^{25}_D$	$K \times 10^5$	$[\alpha]^{25}_D$	$K \times 10^5$	$[\alpha]^{25}_D$	$K \times 10^5$	$[\alpha]^{25}_D$	$K \times 10^5$
days																
0	−158.8		−158.8		−144.8		−144.8		−141.8		−141.8		−139.8		−139.8	
43	−157.3	9.3	−155.5	21	−143.8	6.5	−142.5	16	−138.3	26	−134.8	52	−138.8	7.0	−137.3	17
161	−156.0	4.8	−154.1	8.1	−142.8	3.9	−138.5	12	−132.5	19	−116.8	52	−138.3	2.9	−135.3	8.9
419	−154.8	2.7	−140.0	13	−142.0	2.1	−130.9	11	−108.8	27	−65.3	80	−136.0	2.9	−124.3	12
1162	−145.0	3.4	−106.3	15	−133.8	3.0	−105.8	12	−49.8	39	+2.2		−131.3	2.4	−101.3	12
1555	−139.5	3.6	−94.7	14	−129.7	3.1	−96.7	11	−14.0	65	+6.5		−126.5	2.8	−89.5	12
2078	−132.0	3.8	−67.5	18	−119.2	4.1	−73.5	14	+9.5		+8.0		−119.8	3.2	−68.5	15
2654	−120.0	4.6	−47.0	20	−109.5	4.6	−52.0	17	+10.0		+5.0		−110.5	3.8	−54.5	15

The course of racemization and the velocity constants obtained are recorded in Table I, which is abbreviated from the data collected by the writer.

It is evident from Table I that the effect of temperature and concentration of acid on the rate of loss of optical activity was what might have been predicted: more rapid racemization was caused by the higher temperature and by the more concentrated acid. The first order constants recorded are less regular in the case of the room temperature solutions, owing to the fluctuations in temperature experienced, but in all cases fair constancy was obtained as long as the reaction was chiefly one of simple racemization.   It will

TABLE II

*Summary of Cystine Decomposition in Acid after 90 Months**

Solution	Temperature	Percentage of optical activity remaining	Percentage of cystine remaining (Folin-Marenzi)	Percentage of sulfur converted to sulfate
	°C.			
1.0 per cent cystine in 0.5 N HCl	Room	75.5	77.3	7.65
	38	29.6	49.4	36.8
1.0 per cent cystine in 2.5 N HCl	Room	75.7	77.3	3.46
	38	36.0	59.2	15.0
0.5 per cent cystine in 20 per cent HCl (6 N)	Room	0	0	5.30
	38	0	0	8.14
1.0 per cent cystine in 2.5 N H₂SO₄	Room	79.0	100	
	38	39.0	100	

* The percentage of amino nitrogen remaining, determined according to the Van Slyke method, was 100 in each determination.

also be noted that there is no distinction between H_2SO_4 and HCl solutions of the same normality during this stage of the reaction.

The rapid increase in velocity in both 6 N HCl solutions as well as the surprising development of a dextrorotation indicates some decomposition.   Examination of both 6 N HCl solutions showed a complete lack of any cystine as indicated by the Folin-Marenzi test while the lead plumbite test for "labile sulfur" was also negative. Similar examination of the other solutions showed that correspondingly smaller proportions of the cystine had been lost except in the case of the two H_2SO_4 solutions in which the Folin-Marenzi method gave a quantitative return of all cystine originally weighed in.

There was no evidence of loss of ammonia, and Van Slyke determinations of free amino nitrogen gave (3 minute reaction) values corresponding to the total amount of original cystine.

Somewhat irregular amounts of the sulfur were converted to sulfuric acid. This diversion of a part of the sulfur occurs to a larger extent when the whole reaction is slower but appears to be partly a matter of temperature. It is, no doubt, the result of direct atmospheric oxidation of some intermediate substance and proceeds further the more protracted the whole process is. The solutions all gave negative nitroprusside tests. There was, moreover, no indication of any formation and loss of hydrogen sulfide as total sulfur determinations by the Benedict-Denis method on the hydrochloric acid solutions gave amounts of $BaSO_4$ corresponding quantitatively to the original amounts of cystine. In Table II are summarized the conditions of these solutions after 2654 days.

Cysteic acid has been isolated as the principal oxidation product by evaporation of the solutions in 20 per cent HCl *in vacuo* to a heavy syrup and precipitation with about 20 volumes of absolute alcohol. The crystals obtained correspond to the rhombic octahedra obtained by Shinohara (1) and others. They gave, after drying at 80° *in vacuo*, the following micro analysis.[1]

4.136 mg.: 1.51 mg. H_2O and 3.22 mg. CO_2
4.217 " : 0.304 cc. N_2 at 26° and 757 mm.
5.803 " : 7.75 mg. $BaSO_4$

	Found per cent	Calculated for $C_3H_7O_5NS$ per cent
H	4.09	4.14
C	21.23	21.28
N	8.19	8.27
S	18.35	18.93

The cysteic acid showed a specific rotation of $[\alpha]_D^{25} = +6.0°$ and melted (with decomposition) at 268°. Titration of the sulfonic acid group with alizarin indicated a purity of 95.3 per cent as cysteic acid (compare Clarke and Inouye (2) and Shinohara (1)).

The amount of cysteic acid obtained from a sample of the 20

[1] We are indebted to the kindness of Dr. H. T. Clarke of the College of Physicians and Surgeons, Columbia University, for having this analysis made in his laboratories.

per cent HCl solution (38°) corresponded to 78 per cent of the original cystine present.

DISCUSSION

The reaction here reported appears to be one of direct atmospheric oxidation, catalyzed either by intermediate formation of halide compounds or even perhaps by the intermediate formation of minute amounts of the free halogen. Further catalysis may also result from the presence of the small amounts of such metals as iron and copper which these solutions contained and which such solutions always contain unless made up from specially purified reagents.

The diversion of some of the sulfur to inorganic sulfate, together with quantitative recovery of the free amino nitrogen by the Van Slyke method, makes necessary the assumption that small amounts of some amino compound other than cysteic acid, containing a smaller proportion of sulfur, are formed.

The possibility was considered that the cystine might be slowly undergoing the Vickery rearrangement (3) with simultaneous oxidation to cysteic acid and reduction to cysteine followed by atmospheric oxidation of the latter to cystine and repetition of the cycle. However, the failure to obtain a positive nitroprusside reaction in any case argues against this hypothesis unless we assume incredibly efficient atmospheric reoxidation of the cysteine at the acidities employed. At present, therefore, direct atmospheric oxidation appears most probable.

The oxidation to cysteic acid appears to proceed independently of the amount of racemization and the resulting cysteic acid is partially racemized as will be noted by the value of +6.0° as compared with the value of +8.2° reported by Friedmann (4) and by Shinohara (1).

Further experiments are in progress to determine the mechanism of the reaction, the effect of various catalysts, etc.

SUMMARY

The rate of racemization of l-cystine in hydrochloric acid and sulfuric acid solutions and the velocity constants for the reaction have been determined for solutions in 0.5, 2.5, and 6 N HCl and in 2.5 N H_2SO_4 at both room temperature and 38°.

In addition to the racemization, the cystine is oxidized by atmospheric oxygen to cysteic acid but this oxidation is confined entirely to the hydrochloric acid solutions. There was no evidence of oxidation in the sulfuric acid solutions.

There is no evidence of any formation of hydrogen sulfide or of any other compound reacting positively with the nitroprusside reagent. There is no evidence of ammonia evolution. The Van Slyke determination gives (in a 3 minute reaction) a yield of amino nitrogen corresponding quantitatively to all of the cystine originally present.

Small amounts of the cystine are oxidized to sulfuric acid but the principal oxidation product is the cysteic acid, partially racemized, which has been isolated to the extent of about 78 per cent of the original cystine present and identified by analysis, optical activity, titration, and "melting point." The reaction appears to be one of atmospheric oxidation.

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THE DETERMINATION OF METHIONINE IN PROTEINS

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INTRODUCTION

Methionine was discovered in 1921 by Mueller (1) in the course of his search for a growth-promoting factor found in some proteins. The subsequent history of its synthesis and resolution is to be found in the excellent review by Vickery and Schmidt (2). So far no methods for its determination in proteins have been published although some papers relative to the subject have appeared.

The interest of the early workers (3–6) centered about the problem of the methylation of proteins. They subjected their material to analysis for methoxy and methylimide before and after treatment with various methylating agents. Some interesting results were obtained regarding the groups in the proteins which could take up the methyl radical.

The significance of this work was greatly changed, however, by two discoveries: first, Burn (7) showed that amino acids and other common substances which did not contain the methylimide group, decomposed during the determination, yielding volatile iodides and giving a precipitate of silver iodide in the absorber, and secondly, the discovery (8) that Mueller's new amino acid contained a methylthiol group which cannot be differentiated from methoxy by the techniques used.

The isolation of methionine from enzymatic digests of protein removes any doubt that methionine is a primary protein decomposition product and not the result of secondary reactions (9).

It is clear from these papers that proteins must contain the methylthiol group but the presence of methoxyl and methylimide seems doubtful. These groups are all determined by similar methods; namely, the reaction of hydriodic acid which liberates

methyl iodide. This volatile iodide is blown through an absorption chain by CO_2 and the iodide determined by gravimetric or volumetric means (10, 11). Methylimide is differentiated from the others by conducting the reaction at high temperature (230°) after completion of the reaction at 130°.

Barger and Coyne (8) have used this method to establish the presence of the methylthiol group in methionine and Freudenberg, Dirscherl, and Eyer (12) showed that crystalline insulin does not contain methionine. The reaction has found its greatest usefulness in the study of the structure of the sugars after methylation.

In applying this method to proteins, one must observe caution in the interpretation of results since these compounds are still so poorly characterized. It is known that glycerol from fats decomposes and yields a volatile iodide under these conditions. Ethyl alcohol and ether, which are used in purifying the proteins, also yield volatile iodides and, therefore, must be completely removed. It is possible that the carbohydrate fraction of some proteins may be methylated and thus yield additional quantities of methyl iodide and finally there may be other methylated amino acids besides methionine present in the protein molecule.

In spite of all these hazards we have proceeded to subject proteins to this analysis with the hope of establishing the methionine content. The procedure selected was essentially that of Pollak and Spitzer (10). We have confirmed their observation that the precipitate in the absorber is not all AgI since a portion may be dissolved in nitric acid. When this portion is reprecipitated as AgI the combined weight is equivalent to the methionine taken.

In order to eliminate the tedious treatment of the precipitate we tried a volumetric estimation of the silver remaining in the filtrate from the AgI precipitate and the results were equally satisfactory. This modification so simplified the method that we have adopted it and incorporated it into the basic procedure.

The method as we have used it follows. Fig. 1 shows the apparatus used.

About 0.5 gm. of protein is weighed and transferred to the digestion flask and a small piece of porcelain added to insure quiet boiling. 10 cc. of pure HI (specific gravity 1.7) are added and the flask is connected to the condenser to which one wash bottle and two absorbers are attached. The wash bottle contains 20 per cent

CdSO$_4$ acidified with H$_2$SO$_4$ and 1 cc. of red phosphorus suspension added. This removes any H$_2$S, I, and HI which might come over from the flask. The absorbers contain 10 cc. of a solution of AgNO$_3$ in absolute alcohol. This solution is prepared by boiling 500 cc. of absolute alcohol and 8 gm. of silver nitrate on a reflux for half an hour and allowing the solution to stand in the sunlight for 2 days. It is then filtered through very fine paper and kept in a brown bottle. Washed CO$_2$ is then bubbled through the appa-
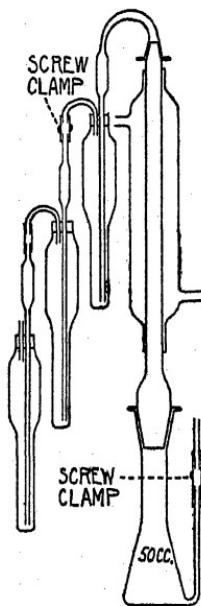
FIG. 1. Apparatus for methionine determination showing the flask, wash bottle, the two absorbers, and the two screw clamps by which the gas bubbling can be regulated in rate of intake and outflow.

ratus from a tank under a pressure of about 2 pounds. (The gas is washed with AgNO$_3$ and H$_2$SO$_4$.) The rate of bubbling is regulated by a screw clamp on the inlet to the flask. The mixture in the flask is brought to boiling with a small flame and the rate of outflow of gas is then regulated by a second screw clamp between the wash bottle and first absorber. The rate of bubbling should be just fast enough so that the bubbles can be counted.

Water at about 60° is run through the condenser jacket. About

90 per cent of the methyl iodide comes over in the 1st hour but the remainder usually takes several hours more. We have allowed the apparatus to run all night (about 15 hours).

The absorbers are then rinsed carefully into 50 cc. beakers and heated on the water bath until the volume is reduced to about 10 cc. The solution is transferred to a 50 cc. volumetric flask, diluted to the mark, and filtered on a dry funnel with very fine

TABLE I

Analysis of Purified Proteins

	Methionine		Methionine
	per cent		per cent
Ovalbumin	4.57	Jones proteins*	
Edestin	2.07	Conavalin jack bean	1.81
Fibrin	2.40	α-Globulin adsuki bean	2.37
Casein	3.53	β-Globulin " "	1.22
Merck's albumin	5.29	α-Globulin tomato seed	3.14
Gortner proteins*		β-Globulin " "	1.72
Fibrin	2.37	α-Globulin Lima bean	2.31
Gliadin	2.03	β-Globulin " "	1.84
Zein	2.21	Glycinin soy bean	1.84
Monococcumin	3.04	Arachin	0.54
Dicoccumin	2.78	Coconut globulin	2.05
Speltin	2.61	Lactalbumin	2.63
Sorghumin	1.76	Stizolobin	1.94
Kafirin	1.61	Zein	2.35
Teozein	3.25	Casein	3.25
Durumin	2.39	Halibut muscle	3.98
Secalin	1.51	Beef muscle	3.66
Hordein	2.24	Shrimp muscle	3.41
Sativin	3.93	Gelatin (Swift)	0.97
Casein	3.36		

* The author wishes to express his gratitude to Dr. R. A. Gortner and to Dr. D. B. Jones for supplying samples of these purified proteins.

paper. 5.0 cc. of the filtrate are then titrated with 0.02 N KSCN after the addition of 2 cc. of HNO_3 and 2 cc. of saturated solution of ferric alum. A micro burette is used.

A blank determination is run as above, omitting the protein, giving the amount of $AgNO_3$ available. The difference between the blank and the determination with protein gives the amount of AgI precipitated by the CH_3I derived from the protein.

The method was standardized with pure synthetic methionine[1] and gave on the average 97.6 ±1.4 per cent recovery.

Eleven amino acids were subjected to analysis by the technique described abcve and all were negative. This is important in view of Burn's finding that most of the amino acids do react in the methylimide technique.

Table I gives the results of the analysis of some purified proteins.

In the mixture of proteins composing Merck albumin there is apparently one which has a higher percentage of methionine than egg albumin.

We were fortunate in having three samples of casein and two of fibrin and zein prepared in different laboratories. The results show excellent agreement.

The α-globulins show at least twice as much methionine as the β-globulins of the bean proteins, and the animal proteins are higher in methionine than the vegetable proteins.

DISCUSSION

We have already pointed out the dangers involved in assuming that the methyl iodide liberated is a measure of the methionine only.

In defense of this assumption we submit that no one has yet isolated an amino acid containing the methoxyl group and none of the amino acids which we have tried, excepting methionine which includes representatives of each of the various classes of amino acids, yields a volatile iodide under the conditions of this analysis. The carbohydrates associated with some proteins are probably not methylated. Finally, when the figures for methionine thus obtained are calculated as sulfur, this fraction exactly supplements the fractions of sulfur present in the proteins as sulfhydryl and disulfide. This we will present in the following paper. On the basis of this complete recovery of the sulfur of proteins we believe our assumption is warranted.

SUMMARY

A technique is described for determining the volatile iodide arising from proteins treated with hydriodic acid.

[1] This was purchased from Dr. C. S. Marvel, University of Illinois, Urbana.

The method gives nearly theoretical figures when pure methionine is subjected to analysis.

The methionine content of proteins subjected to this procedure ranges from 0.5 to 5.0 per cent. Certain difficulties of interpretation of the results are discussed.

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THE SULFUR DISTRIBUTION IN PROTEINS

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INTRODUCTION

The total sulfur in almost all proteins is considerably greater than the sulfur contained in the cystine fraction. This fact was emphasized by Osborne (1) who had only the lead blackening reaction for cystine on which to base his calculations. However, improvements in the methods for determining cystine in proteins has not altered the fundamental relationships pointed out in this early work. If the figures given by Sullivan and Hess (2) are accepted, only 36 per cent of the sulfur of a group of proteins can be accounted for as cystine. If the Folin and Looney figures are used, the per cent recovery is raised to 53 per cent and the corresponding figure for the Folin and Marenzi technique is 58 per cent. The author's gasometric procedure (3) accounts for only 46 per cent of the sulfur as sulfhydryl and disulfide, according to the figures in Table I.

The condition is somewhat different in the keratins studied by Rimington and his coworkers (4). They have made a very careful analysis of various wools and animal hairs and find that these tissues contain enough cystine to account for nearly all of their sulfur.

The problem of accounting for all of the sulfur in proteins was greatly aided by the discovery of methionine by Mueller (5) although few attempts to determine this sulfur fraction have been made (see preceding paper).

In the present work we have attempted to account for all of the sulfur in a group of purified proteins by determining sulfhydryl + disulfide and methionine sulfur. The results were entirely satisfactory and leave no doubt that this distribution is adequate

TABLE I

Analysis of Purified Proteins

The values are given in per cent. All calculations are on a moisture- and ash-free basis.

	Total S	Cystine S—H + S—S	Methionine	S—H + S—S	Methionine	Found
				Total S		
Ovalbumin	1.60	2.27	4.57	37.8	61.4	99.2
Edestin	0.99	1.79	2.07	47.8	44.6	92.4
Fibrin	0.97	1.58	2.40	44.0	53.6	97.6
Casein	0.90	0.65	3.53	19.3	84.2	103.5
Merck albumin	1.83	2.52	5.29	36.9	62.1	99.0
Gortner proteins						
Fibrin	0.97	1.64	2.37	45.0	52.5	97.5
Gliadin	0.99	2.74	2.03	73.7	44.1	117.8
Monococcumin	1.14	2.23	3.04	52.0	57.4	109.4
Dicoccumin	1.20	2.65	2.78	59.4	49.8	109.2
Speltin	1.20	2.71	2.61	59.6	46.8	106.4
Sorghumin	0.56	0.69	1.76	32.9	67.6	100.5
Kafirin	0.59	1.02	1.61	46.2	58.6	104.8
Durumin	1.05	2.34	2.39	59.5	49.0	108.5
Secalin	1.26	2.97	1.51	62.9	25.8	88.7
Hordein	0.90	1.72	2.24	51.0	53.5	104.5
Sativin	1.63	3.65	3.93	59.8	51.8	111.6
Casein	0.80	0.66	3.36	22.1	90.2	112.3
Jones proteins						
Conavalin	0.51	0.36	1.81	18.9	76.2	95.1
α-Globulin adsuki bean	0.88	1.27	2.37	38.5	57.9	96.4
β-Globulin " "	0.40	0.42	1.22	27.9	65.5	93.4
α-Globulin tomato seed	0.97	1.42	3.14	39.1	69.6	108.7
β-Globulin " "	0.79	1.56	1.72	53.0	46.8	99.8
α-Globulin Lima bean	1.15	1.98	2.31	46.0	43.2	89.2
β-Globulin " "	0.35	0.54	0.73	41.2	44.7	85.9
Glycinin soy bean	0.84	1.69	1.84	53.7	47.2	100.9
Arachin peanut	0.42	1.33	0.54	84.3	27.6	111.9
Coconut globulin	1.04	2.09	2.05	53.0	42.3	95.3
Lactalbumin	1.56	3.77	2.63	64.5	36.3	100.8
Zein	0.93	1.58	2.35	45.3	54.1	99.4
Casein	0.83	0.56	3.25	17.7	84.2	101.9
Halibut muscle	1.16	1.85	3.98	42.5	73.7	116.2
Shrimp "	1.25	1.58	3.41	32.5	58.6	91.1

for characterizing the sulfur of this group of proteins. In certain other proteins the preformed sulfate must also be considered.

Methods

Total sulfur was determined by peroxide fusion in a Parr bomb (6).

The sulfur present as sulfhydryl and disulfide was determined by the author's gasometric technique (3). These fractions were calculated as cystine though it was recognized that they might also have arisen from cysteine and thiohistidine. The evidence for the presence of these latter amino acids in the protein molecule is still far from convincing.

A few changes have been introduced since the publication of the original method. In the first place, zinc has been substituted for Devarda's alloy, since this preparation contains copper and during the reduction of disulfide becomes reduced and subsequently uses up some of the iodine which is added to the mixture.

The time allowed for action of zinc has been lengthened to make sure of complete reduction of disulfide to sulfhydryl and this necessitated the introduction of more sodium hydroxide in the hydrazine solution which is used in the Van Slyke manometric chamber to titrate the iodine. The hydrazine solution now contains 1 volume of a saturated solution of hydrazine sulfate and 3 volumes of saturated sodium hydroxide. With this mixture the zinc hydroxide, which first precipitates when the sample is introduced, is completely dissolved with very little difficulty.

The proportions of digest to iodine have been modified to increase the accuracy of the method. The mixture used in the present work contained 6 cc. of protein digest, 2 cc. of concentrated HCl, and 2 cc. of iodine in KI. This change, of course, makes necessary a change in the factor used to convert gas pressure to mg. of cystine. It was thought advisable to determine this factor each day with a standard cystine solution rather than to rely on a precalculated factor as recommended before.

An observation of some interest was made on old solutions of reduced cystine which had stood for some weeks over zinc. These showed the same reducing power as did freshly reduced cystine solutions.

1.0 gm. of protein was hydrolyzed with 20.0 cc of 20 per cent

HCl by boiling in a reflux for 15 hours. The flask was rinsed into a 25 cc. volumetric flask and diluted to the mark with 20 per cent HCl. The digest was reduced with zinc, filtered, and the total sulfhydryl determined.

Methionine was determined by the method published in the preceding paper.

Results

In order to test the methods used we have added various amino acids to gelatin and subjected the mixture to analysis (Table II).

TABLE II

Analysis of Gelatin and Mixture

The values are given in per cent.

	Total S	BaSO₄*	Cystine			Total S				
			S—H†	S—S	Methionine	Sulfate	S—H	S—S	Methionine	Found
Gelatin.................	0.47	0.77	0.15	0.33	0.97	22.5	8.5	18.9	44.4	94.2
Mixture.................	1.91	0.65	1.46	2.79	2.92	4.7	20.6	39.4	33.1	97.8
Amino acids.............	1.47		1.33	2.49	2.05		24.1	45.2	30.0	99.3
" " (calculated)..	1.49		1.99	1.99	1.99		35.7	35.7	28.7	100.1

* Gelatin contains oxidized sulfur which may be precipitated from hydrolysates with BaCl₂.

† The S—H fraction was determined separately and represents the reducing power of the digest before treatment with zinc.

The mixture contained:

	gm.		
Gelatin...............	0.9000	Cysteine HCl..........	0.0262
Methionine............	0.0200	Tyrosine.............	0.0200
Cystine..............	0.0200	Tryptophane..........	0.0200

The figures for amino acids alone were found by subtracting 89.3 per cent of the corresponding gelatin figures from those of the mixture.

It will be observed' that some of the cysteine became oxidized to cystine during the manipulations. It is because of this partial oxidation of sulfhydryl to disulfide that we have thought it best to

report the two fractions together. The presence of tyrosine and tryptophane, two amino acids which give most trouble in some other methods, seems not to affect the results in the slightest here.

The combined fraction of sulfhydryl and disulfide sulfur is difficult to compare with cystine figures from the literature since the compounds composing this fraction are not yet identified and therefore we do not know how the specific color reagents would respond to them. On general principles we should expect these figures to be higher than those reported by others since the iodine oxidation is incapable of differentiating cystine from other disulfides (7). The figures published by Jones (8), using the Folin and Looney method, are about 87 per cent of those given in this paper and those published by Sullivan, using his more specific color reaction, are about 73 per cent.

The proportion of sulfhydryl to disulfide is not a constant in any particular protein. It seems to depend on several ill defined conditions obtaining in the digestion flask chief among which is probably the amount of humin formation. Duplicate hydrolyses do not give exactly the same distribution, although the total sulfhydryl figures obtained after reduction of the digest with zinc check very well. When it is remembered that sulfhydryl is readily oxidized by the air even in 20 per cent HCl this finding is to be expected.

There are several considerations which tend to support the hypothesis that the figures for cystine obtained by other methods are too low. First, methods requiring decolorization are subject to losses of cystine adhering to the decolorizing agent or to the humin removed (9). There is also evidence that the humin itself contains cystine or its decomposition products (10). Most workers have attempted to compensate for this loss by applying a correction factor obtained by putting pure cystine solutions through the decolorizing procedure. Such solutions, of course, contain no humin. We have used a procedure which does not require decolorization.

Any procedure which requires neutralization of the acid digest and subsequent development of a color with a highly specific

reagent is likely to give low values due to alkaline decomposition of the cystine. In the gasometric technique no alkali is introduced until after the action of the iodine is complete.

The Sullivan procedure, which gives minimal values, would fail if all the linkages between cystine and other amino acids were not broken, whereas, all other procedures would include this fraction.

Shinohara (11) has recently pointed out that iodine can oxidize disulfides to sulfonic acids. If this occurred in our mixtures, it would make our results too high. However, pending an investigation of the question, we submit that our mixtures contain more acid than those reported by Shinohara and also we use mixtures that are not over 15 minutes old. Both of these conditions make it seem unlikely that the reactions mentioned above can occur.

SUMMARY

A system of analysis has been developed for fractionating the sulfur of proteins into sulfhydryl + disulfide, and methionine sulfur.

The methods have been standardized with pure amino acids and by recovery from gelatin digest.

The sulfur distribution of thirty-two purified proteins is given in Table I, showing 101.5 ±6.6 per cent recovery of the sulfur.

The range of sulfhydryl + disulfide sulfur was 17.7 to 84.3 per cent of the total sulfur, and the methionine sulfur from 26.0 to 90.0 per cent.

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RELATIONSHIPS BETWEEN THE ACTIVATION OF PAN-CREATIC LIPASE AND THE SURFACE EFFECTS OF THE COMPOUNDS INVOLVED

THE MECHANISM OF INHIBITION AND ACTIVATION*

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Definite relationships have been found between the structure of organic compounds and their inhibiting effects upon liver esterase. These relationships may be shown for both the hydrocarbon portion of the molecule (1) and the substitution groups (2).

In the present communication a report is given of the effects upon pancreatic lipase of representative compounds chosen from those studied in relation to liver esterase. The latter enzyme is fairly specific for the hydrolysis of esters of monoatomic alcohols, while the former appears to be fairly specific for the hydrolysis of glyceryl esters of fatty acids. It was thought that an investigation of this type would demonstrate more clearly the differences between the two related enzymes, and would also provide information regarding their chemical nature and function.

The data obtained during this investigation indicated that the surface relations between an enzyme and the surrounding solution were of fundamental importance to an interpretation of the mechanism of the observed phenomena. Accordingly, surface tension measurements were made for the solutions used, and a tentative explanation has been suggested for the phenomena observed in studying both inhibition and activation.

Willstätter and coworkers (3, 4) have found that calcium oleate, bile salts, and albumin activate pancreatic lipase in an alkaline medium and inhibit it in an acid medium. In the case of the bile

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* Contribution No. 245 from the Department of Chemistry, University of Pittsburgh.

salts it was shown that either gastric or pancreatic lipase could be activated only after a certain degree of purification of the enzymes had been attained (5). At higher concentrations the bile salts showed decreased activation (6). Proteins appeared to inhibit the hydrolysis of tributyrin, though certain amino acids and peptides accelerated it (6).

It has also been reported that the degree of dispersion of the substrate has very little influence on the rate of lipase action (7), and that bile salts activate the hydrolysis of soluble esters by lipase (8). In contrast, other work has indicated that bile salts inhibit liver esterase (9). We believe that the present investigation throws some light upon the apparent discrepancies reported in the literature.

<div align="center">EXPERIMENTAL</div>

<div align="center">*Preparation of Materials*</div>

Fresh lamb pancreas was reduced to a fine dry powder by the acetone defatting and dehydrating method of Willstätter and Waldschmidt-Leitz (10). Stored in a refrigerator, the material maintained full lipase activity during a 6 month period.

Extracts were made from this preparation by the same method employed for liver esterase (1). In the preparation of esterase the supernatant liquid after acetic acid precipitation was found to contain most of the enzyme; but in the case of the lipase the acetic acid precipitate contained practically all the enzyme. This indicates that the isoelectric points of the proteins related to the two enzymes are distinctly different.

The acetic acid precipitate containing the lipase from 0.25 gm. of powder was finally dissolved in 0.025 N NH$_4$OH, made neutral to brom-thymol blue (outside indicator) with acetic acid, and diluted to a final volume of 25.0 cc.

Lipase extracts prepared as above were decidedly more active than those prepared by water or glycerol extraction of the dry powder. The extract showed but little activity when first made, but after standing a few days in a refrigerator, it became slightly cloudy, and in this state it exhibited its maximum lipase activity. After 10 days or more, microorganisms may appear, and the material must then be discarded.

The phenol red indicator was prepared by dissolving 0.1 gm. of

the solid in 1 liter of water. Samples of the organic compounds used in the previous investigations (1, 2) were employed in the present work. A saturated aqueous tributyrin solution, freshly prepared when needed, was used for the lipase substrate.

The bile salts were separated from Merck's technical sodium taurocholate by a method based on the neutral lead acetate precipitation of soluble glycocholates, and the basic lead acetate precipitation of soluble taurocholates (11). The lead salts were dissolved in warm 95 per cent alcohol, treated with H_2S, the solution filtered, and the free acids purified by reprecipitation from absolute alcohol by addition of absolute ether.

Procedure

Methods for measuring the extent of hydrolysis by lipase, in which substrate emulsions of glycerides or oils are employed, were

TABLE I

Activation of Lipase by Amyl Alcohol

Flask No.	1	2	3	4	5	6
Amyl alcohol, *mols* × 10^{-6}	0	45.4	90.8	136.2	181.6	227.0
NaOH, *cc*	0.39	0.43	0.45	0.46	0.48	0.49
Activation, *per cent*	0	10	15	18	23	26

not satisfactory for the present investigation, since it is difficult to reproduce emulsions exactly, and the altered surface relations, which the presence of another phase would introduce, would render an interpretation of the results more difficult. For this reason, a simple convenient method for determining the degree of hydrolysis has been employed by which the enzyme is suspended in a true solution of the substrate with the compound whose effect is to be measured.

A run was made in the following manner. 50 cc. of saturated tributyrin solution were added to each of six 125 cc. Erlenmeyer flasks. With the first one for a blank, increasing quantities of activator solution were added to the others. Water was introduced until the final volume in each flask was 60 cc. Then 0.2 cc. of lipase extract was added to each flask. After corking, they were kept at 37° for 2 hours. At the end of that time 25.0 cc. of

95 per cent alcohol were placed in each flask to stop the enzyme action.   The solutions were then titrated with 0.07 N NaOH, 4 drops of phenol red being used in each flask as the indicator.   The titration was performed with a 10 cc. burette accurate to 0.05 cc. and capable of estimation to 0.01 cc.   The results of a characteristic experiment are given in Table I.

In the case of resorcinol, methyl red was used as the indicator since this compound seems to have a buffering effect in the pH range where phenol red changes color (6.8 to 8.4).

The surface tensions of the activator solutions were measured by the capillary rise method.   For this purpose, a long test-tube containing the solution whose surface tension was to be measured
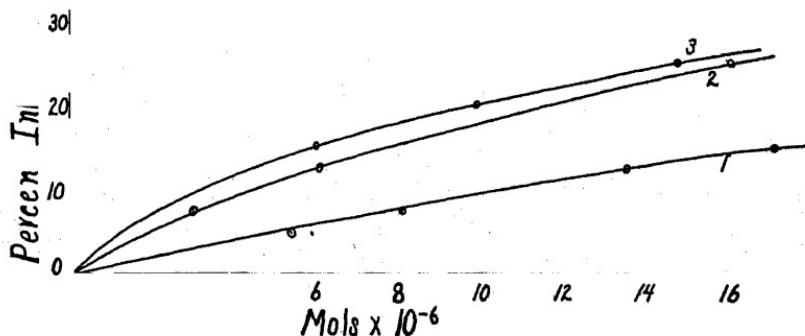
Fig. 1. Inhibition of esterase by organic compounds.   Curve 1, sodium taurocholate; Curve 2, sodium glycocholate; Curve 3, resorcinol.

was placed in a thermostat maintaining a constant temperature of 20°.   The capillary tube was fixed so that its lower end dipped below the surface of the solution in the test-tube.   The level of the liquid in the tube was kept well below the level of the water in the thermostat.   The height to which the solution rose in the capillary was measured by a cathetometer accurate to 0.01 cm.

The surface tensions in dynes per cm. were calculated from the equation

$$s = \tfrac{1}{2} \, hdgr$$

in which s represents surface tension, h height in cm. of the liquid column above the surface of the solution in the test-tube,

d density in gm. per cc., g gravitational constant, and r radius in cm. of capillary bore. r was calculated from observation of h for pure water, and the known value of s for water at 20°. The densities of all the solutions were taken as unity since they were so dilute as to render the difference beyond the experimental error of the measurements involved.

The inhibition of bile salts and resorcinol upon liver esterase was determined by the method described in a previous paper (1). The results were included in the present communication to complete the comparison of the effects of compounds upon esterase and lipase (Fig. 1). The respective inhibition numbers were as follows: sodium taurocholate 15 (extrapolated), sodium glycocholate 27, resorcinol 30.

DISCUSSION

The activating effects of different types of organic compounds on pancreatic lipase are shown in Fig. 2. It will be seen that representative normal primary alcohols, saturated and unsaturated cyclic compounds, and two substituted pentanes are included in the study.

Experiments with isomers of amyl alcohol showed them to have too little activating effect to be measured by the method employed.

Since the primary alcohols become less soluble, and their activating effect greater, as the molecular weight increases, all alcohols higher in the series than octyl were too insoluble to show any activating effect, while alcohols lower than amyl, though sufficiently soluble, were too poor as activators to exhibit an effect. Likewise, the influence of the pentane derivatives, other than those given in Fig. 2, could not be determined by the present method.

The bile salts showed an inhibition rather than activation of the lipase acting on saturated tributyrin. This would be expected from the work of Willstätter and coworkers (3, 4) who showed that activation occurs only in an alkaline medium, while inhibition results in an acid solution. In this case, the pH soon fell below 7.0 as the experiment proceeded. It was found that sodium glycocholate inhibited more than sodium taurocholate. However, with an emulsion of 1 volume of 1 per cent sodium oleate with 4 volumes of olive oil as substrate, the bile salts activated the

enzyme; the glycocholate was a better activator than the tauro-cholate.

It has been reported by Murray (12) that certain ketones, aldehydes, secondary alcohols, and sodium salts inhibit the activity of pancreatic lipase. It is possible that a significant amount of esterase may have been present with the lipase used, and a further difference lies in his use of higher concentrations of added organic compounds. It is known that an activator may become an inhibitor, if the concentration of the substance is increased beyond certain limits.
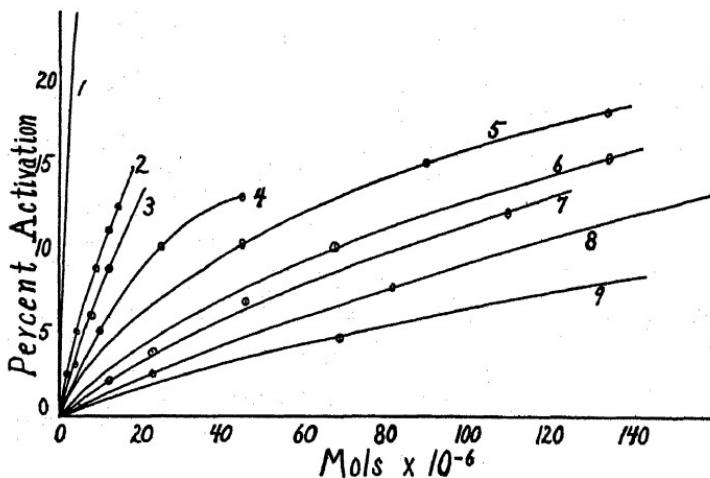
Fig. 2. Activation of lipase by organic compounds. Curve 1, hexyl resorcinol; Curve 2, octyl alcohol; Curve 3, amyl iodide; Curve 4, hexyl alcohol; Curve 5, amyl alcohol; Curve 6, phenol; Curve 7, caproic acid; Curve 8, cyclohexanol; Curve 9, resorcinol.

The surface tensions of the activator solutions at different concentrations are given in Fig. 3. It is evident from reference to Fig. 2 that the more a compound lowers the surface tension of water, the better is its activating effect upon lipase. To this general relationship there is exception in the case of phenol, which does not have as great an effect upon surface tension lowering as would be expected from its relative activating influence. There also seems to be a relationship between the order of surface tension lowering and the order of inhibiting effect of the same compounds upon esterase (1, 2).

It appears to the authors that the most reasonable interpretation of the results obtained is that there are at least two opposing tendencies operating when such foreign substances are added to a solution containing enzyme and substrate. One tendency is to produce an inhibition of the enzyme due to a combination of the foreign compound, either chemically or physically, with the enzyme to give an inactive complex; or, a combination of the added compound with the substrate may occur. The other tendency is to produce an activation of the enzyme by making the enzyme and substrate molecules more accessible to one another. This
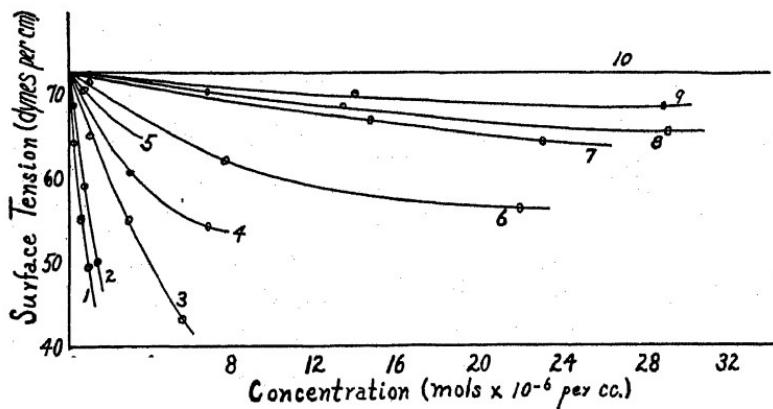
FIG. 3. Effect of concentration on surface tensions of solutions. Curve 1, hexyl resorcinol; Curve 2, octyl alcohol; Curve 3, sodium glycocholate; Curve 4, sodium taurocholate; Curve 5, amyl iodide; Curve 6, amyl alcohol; Curve 7, caproic acid; Curve 8, cyclohexanol; Curve 9, phenol; Curve 10, resorcinol.

is accomplished by lowering the interfacial tension between them by the addition of a foreign substance.

The mechanism of inhibition has previously been discussed but the way in which a substance may lower the interfacial tension between an enzyme and substrate to give activation has been given little consideration. From Gibb's theorem,

$$U = \frac{-c}{RT} \frac{ds}{dc}$$

in which U represents excess concentration in boundary layer per sq. cm., c concentration of solute in the liquid, R gas constant,

s surface tension, T absolute temperature, it is seen that if a substance progressively lowers the surface tension of the solution as more of it is dissolved, $\dfrac{ds}{dc}$ will be negative, and hence U will become positive. In other words, the boundary layer will be richer in the dissolved substance than the bulk of the solution.

It is thus apparent that substances, such as the activators employed, which lower surface tension will concentrate at the surface of the enzyme; but the activating effect is produced as a secondary result. Antonow (13) has shown that the interfacial tension between two liquid phases is equal to the difference in their surface tensions:

$$S_i = S_a - S_b$$

TABLE II

Order of Effect of Organic Compounds

Surface tension lowering	Activation of lipase	Inhibition of esterase
Hexyl resorcinol	Hexyl resorcinol	Hexyl resorcinol
Octyl alcohol	Octyl alcohol	Octyl alcohol
Sodium glycocholate	Amyl iodide	Amyl iodide
" taurocholate	Hexyl alcohol	Hexyl alcohol
Amyl iodide	Amyl "	Amyl "
" alcohol	Phenol	Phenol
Caproic acid	Caproic acid	Resorcinol
Cyclohexanol	Cyclohexanol	Sodium glycocholate
Phenol	Resorcinol	" taurocholate
Resorcinol		Cyclohexanol

where S_i is the interfacial tension, S_a surface tension of the aqueous phase, and S_b surface tension of the other phase. From this, substances that lower the surface tension of a solution should cause a decrease in interfacial tension between the solution and an immiscible liquid. This apparently affords an explanation of the activation, since the activator, though concentrating on the enzyme surface itself, lowers the interfacial tension between the enzyme and substrate.

Where this interpretation is applied to the liver esterase (it being assumed the compounds studied showed inhibition of the enzyme by the formation of inactive complexes), the inhibiting effect was made stronger by the fact that surface actions caused the

compounds to concentrate at the enzyme surface, thus enhancing combination. Unless there is a tendency to combine with the "active centers" of the enzyme, this type of surface concentration would not be expected to produce an inhibiting effect.

In the case of pancreatic lipase, significant amounts of inactivating complexes were not formed with the compounds investigated, so that the surface effect was the only or dominating one, and activation occurred.

This too affords a possible explanation of the fact that bile salts inhibit liver esterase though they activate pancreatic lipase.

A comparison of the effects of the various compounds considered is given in Table II. It will be observed that the best inhibitors for the esterase are the best activators for the lipase, and also lower the surface tension in greatest degree. The exception of the bile salts, which do not show as marked inhibiting properties as would be expected, is possibly in part due to their tendency to combine with fatty acids (14). Such a combination with, and hence removal of, butyric acid would hasten the hydrolysis, since accumulation of the end-products of the reaction causes a retardation.

The only other exception is that of phenol and resorcinol, which are, however, weakly active, with effects that are not clear cut. Their slightly acidic character may have interfered with a satisfactory titration.

SUMMARY

The inhibiting effects of bile salts and resorcinol upon liver esterase have been measured.

A method has been described for the measurement of the activation of pancreatic lipase by organic compounds, in which the enzyme and the surrounding solution are the only two phases present.

The activating effects of a number of compounds upon pancreatic lipase have been measured and compared to the inhibiting effects of the same substances upon liver esterase. In general the compounds which exhibited the most inhibition of esterase were the ones which activated lipase to the greatest degree.

The surface tensions of a number of solutions of the compounds studied have been determined at different concentrations, and the results used to present a theory of the possible mechanism of the inhibiting and activating effects observed.

Further distinctions between lipases and esterases have been demonstrated, particularly in regard to their precipitation by acetic acid.

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THE DISTRIBUTION OF GLUCOSE IN HUMAN BLOOD

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Glucose is readily soluble and so far as we know readily permeates the red blood cells. Unlike electrolytes, there is no reason to suppose that the conditions which determine the distribution of electrolytes on the two sides of the erythrocyte "membrane" would influence its distribution, so that it is reasonable to believe that the concentration of glucose in the plasma and erythrocytes would be according to the water concentrations of these media. Although there are scores of observations reported in the literature concerning the distribution of sugar between the corpuscles and plasma of the blood of man and various animals, none of them to our knowledge considers this point directly. The earlier investigations have been summarized by Macleod (1) and the more recent ones by Somogyi (2) and others (3, 4). In examining the published data it is obvious in most instances, on the basis of the usual fluid content, that the concentration of sugar in the cell water is a great deal higher than in the water contained in the plasma. However, all of these investigators assumed all of the reducing substance in the blood to be glucose, and this is not the case. The nature and red blood cell permeability of the non-glucose fraction which is not absorbed by yeast is unknown. Since the concentration of this non-glucose-reducing fraction in the erythrocytes has been found (2) on a volume basis to be about 5 times that of plasma, a source of gross error in most blood sugar distribution studies is evident. Somogyi (2) has ruled out this inaccuracy and dealt with true in place of apparent glucose values which had always been used heretofore. This reduced the ratio $\dfrac{\text{Corpuscle sugar concentration}}{\text{Plasma sugar concentration}}$ (by volume) to nearer a point which might be expected from the usual water concentrations of the

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two phases of the blood. But even these figures suggest that in man at least the cell water may contain a higher concentration of sugar than the plasma water. Experiments which suggest that this may be the case with urea have led us to examine this point.

Methods

Samples of venous blood were drawn from a superficial arm vein, with momentary stasis, from a number of normal subjects and patients. A minimal amount of heparin prevented coagulation. A part of each specimen was centrifuged immediately for 12 minutes at 4000 R.P.M. which sufficed for satisfactory packing of the red blood cells. The plasma was quickly withdrawn, the first portion saved, and the last remnant along with the buffy coat and the uppermost layer of packed cells drawn off and discarded as speedily as possible. The red blood cells were well mixed with a stirring rod and roughly measured specimens of the whole blood, plasma, and cells were pipetted into weighed flasks containing weighed amounts of zinc sulfate (1.25 per cent) -sulfuric acid (0.03125 N) solution. These were again weighed to determine the weight of the blood samples and once more after the addition of the sodium hydroxide (0.75 N) for precipitating the proteins. The weight of the mixture was then known, and as the water content of each precipitated mass was determined, the weight of water in which the glucose from a given weight of blood was distributed could easily be calculated. Weighed samples of the whole blood, plasma, and red blood cells were also used for determining their original water content. When the protein-free filtrates were pipetted for the glucose determinations a similar pipetteful of each was weighed and the water loss determined so that the amount of water containing the glucose measured could be known. From the water concentration of the original blood specimens, the diluted mixture after the addition of the protein precipitants and the protein-free filtrate, and with the weight of water used in the glucose analysis, it was then easy to calculate the amount of glucose in the water contained in a given weight of whole blood, red blood cells, or plasma.

The proteins were precipitated from the blood samples by the use of zinc hydroxide in the manner described by Somogyi (5). He concluded that this treatment yields filtrates which contain

substantially no non-sugars when oxidized by copper reagents and gives the "true" blood sugar or results practically the same as those found by fermentation with yeast. Benedict (6) found appreciable amounts of saccharoids or non-fermentable reducing substances with both his new copper reagent, and the Folin-Wu method in such filtrates. In our hands the zinc hydroxide filtrates

TABLE I

Distribution of Glucose between Water of Erythrocytes and of Plasma of Human Blood

Subject and conditions	Glucose per 100 gm.			H_2O per 100 gm.			Glucose per 100 gm. H_2O			Glucose concentration of Plasma H_2O / Cell H_2O
	Whole blood	Plasma	Cells	Whole blood	Plasma	Cells	Whole blood	Plasma	Cells	
	mg.	*mg.*	*mg.*	*gm.*	*gm.*	*gm.*	*mg.*	*mg.*	*mg.*	
Cardiac, 3 hrs. after breakfast.	122	138	101	80.0	91.0	67.7	153	152	149	1.02
Normal, 1 hr. after lunch....	104	118	86	79.4	90.5	66.3	131	130	130	1.00
" 1 " " "	101	121	94	79.5	91.8	70.6	127	132	133	0.99
" 3 hrs. " breakfast.	69	80	59	79.5	91.3	66.2	87	88	89	0.99
" 3 " " " .	80	93	66	77.8	91.3	65.4	103	102	101	1.01
Thyroid adenoma, 2 hrs. after breakfast.................	125	144	102	80.2	91.4	67.1	156	157	152	1.03
Normal, fasting.............	76	91	65	79.2	90.8	66.2	96	99	98	1.01
Diabetic, 4 hrs. after breakfast and insulin...........	36	43	29	80.8	91.0	66.0	45	47	45	1.04
Diabetic, fasting...........	72	85	61	79.0	91.0	66.4	91	94	92	1.02
Normal, 4 hrs. after breakfast.	73	85	59	79.5	93.6	66.2	92	92	89	1.03
" 2 " " lunch....	116	132	99	79.5	90.8	67.4	146	145	147	0.98
" 2 " " "	77	89	66	78.6	90.2	66.5	98	99	100	0.99
" fasting.............	79	90	67	79.2	90.9	68.2	99	99	98	1.01
" "	84	97	70	81.1	91.2	67.4	104	106	104	1.02
" 4 hrs. after breakfast.	91	102	77	78.2	89.8	65.6	116	113	118	0.97

were entirely free from non-fermentable reducing materials when measured with the particular copper reagent which we employed. The glucose concentration of the measured portions of filtrate of known weight was determined by the Shaffer-Hartmann (7) procedure, with a modified copper reagent proposed by Somogyi and Shaffer (8).

The water content of the blood samples was determined by drying for 24 hours at 105° in an ordinary oven followed by 24 hours at 90–100° in a vacuum oven. After this treatment the specimens lost no additional weight following a longer period of drying.

Results

In Table I are presented the results obtained upon fifteen specimens of blood. The distribution of glucose between the

TABLE II

Distribution of Glucose Added in Vitro between Water of Erythrocytes and of Plasma

Sample No.	Conditions	Glucose per 100 gm.			H₂O per 100 gm.			Glucose per 100 gm. H₂O			Glucose concentration of $\frac{\text{Plasma H}_2\text{O}}{\text{Cell H}_2\text{O}}$
		Whole blood	Plasma	Cells	Whole blood	Plasma	Cells	Whole blood	Plasma	Cells	
		mg.	mg.	mg.	gm.	gm.	gm.	mg.	mg.	mg.	
1	3 hrs. after breakfast	69	80	59	79.5	91.3	66.2	87	88	89	0.99
1-A	4 mg. glucose per cc. added *in vitro*	491	558	407	79.1	90.5	65.2	620	617	626	0.99
2	3 hrs. after breakfast	80	93	66	77.8	91.3	65.4	103	102	101	1.01
2-A	4 mg. glucose per cc. added *in vitro*	483	573	395	77.3	90.5	63.9	624	633	618	1.02
3	3 hrs. after breakfast	73	85	59	79.5	98.6	66.2	92	92	89	1.03
3-A	2 mg. glucose per cc. added *in vitro*	344	384	291	79.6	91.2	66.4	432	421	439	0.96
3-B	7 mg. glucose per cc. added *in vitro*	702	800	574	79.3	90.6	65.9	886	882	862	1.02

The blood samples used were all taken from normal subjects.

plasma and erythrocytes in these freshly drawn blood specimens is equal, for the concentrations of glucose in the plasma water and cell water are essentially the same.

In view of this equal distribution of glucose in the blood *in vivo* it seemed of interest to examine the distribution of glucose added *in vitro* to human blood. Specimens of blood were divided into two or more parts. One was treated in the manner we have described. To another part of the specimen a roughly weighed

quantity (2 to 4 mg. per cc.) of powdered C.P. *d*-glucose was added. The blood (usually 30 cc. in a 125 cc. Erlenmeyer flask) was then gently whirled by a rotating swing of the flask for 10 minutes. A portion was then centrifuged and the plasma, cells, and whole blood were treated as before. During the mixing the blood became oxygenated, as judged by the bright red color it acquired. As can be seen from the data in Table II this short period of agitating the blood was sufficient to distribute the added glucose equally between the plasma and cell water just as it exists *in vivo*. The distribution of glucose between the erythrocytes and plasma is apparently a process of simple diffusion and dependent upon the water concentrations of these two media. All of the water, both free and bound, if such exists, appears to be available for the diffusion of glucose.

SUMMARY

Glucose is found in the same concentration in the water contained in the plasma and erythrocytes of freshly drawn human blood and after the addition of glucose *in vitro*. The distribution of glucose between the erythrocytes and plasma is apparently determined by the water concentrations of these media.

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STUDIES ON BIOLOGICAL OXIDATIONS

I. OXIDATIONS PRODUCED BY GONOCOCCI*

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INTRODUCTION

The problem of biological oxidations, which has been the subject of many important contributions during these last years, still remains unsolved, as every theory developed to explain the mechanism of the oxidations performed by the cell has always encountered insurmountable objections. Too often these theories have been the result of experiments performed with but a few types of living cells, and the broad generalizations drawn from these limited findings were subsequently found to be inapplicable when other types of cells were studied.

As the capacity of living matter for developing energy-yielding processes increases with phylogenetic development, it is unquestionable that in order to understand the mechanism of oxygen utilization by cells higher up the phylogenetic scale, where the nature of biological oxidations becomes more delicate and complex, it will be essential to understand the nature of oxidations performed by cells occupying the lowest place in the phylogenetic scale.

The energy required for the maintenance of cell activities comes, as the celebrated studies of Pasteur (1) demonstrated, from two sources: fermentation and oxidation. The cells belonging to the lowest group in the phylogenetic scale are undoubtedly those capable of "vie sans air," in which the energy requirements are met by fermentation processes. We may say, as Wieland (2) has suggested, that it is the greater energy requirement of morphologically and physiologically complex organisms which has introduced atmospheric oxygen into the metabolism of living matter. Since we are concerned with the study of biological oxidations we will discard those fermentative cells and limit our attention to those cells which derive their main energy from oxygen utilization. It is easy to understand why our attention has first been turned to the oxidations produced by bacteria. They belong to a low place in the phylogenetic scale and for this reason their oxidation processes are more amenable to quantitative studies. But, as must be expected, even in this group of organisms there are some cells capable of performing quite complicated oxidations. Callow (3), who was the first to measure the respiration of bacteria, has shown that many of them are able to utilize oxygen even after they have been washed out of the oxidizable substrates surrounding them. This "endogenous respiration" which is beyond the control of the investigator is undoubtedly a great obstacle to the quantitative study of the oxidations performed by bacteria. It was, therefore, essential for us to choose a bacterial organism with an oxidation mechanism as simple as possible and devoid of endogenous respiration. With bacteria possessing such qualities complete studies of their oxidation processes would be possible,—studies to be used as a basis for subsequent researches on the mechanism of cellular oxidations.

Various investigators at the Cambridge School of Biochemistry have studied extensively the oxidations produced by *Bacillus coli*, bacteria which possess the ability to perform a considerable num-

ber of oxidations (56 out of 103 substances investigated). *Sarcina lutea*, bacteria which have been used by Gerard (4), similarly seem to perform a great number of oxidations. Moreover, these cells when washed and suspended in saline solution show a steady oxygen consumption which introduces an error in calculations, since the nature of the substrate oxidized by the cell in such conditions is not known. After numerous tests we found that gonococci constitute an excellent material because of the relatively small number of substrates which this cell is able to oxidize and the absence of endogenous respiration. As is known, glucose is the only sugar which is fermented by gonococci, and since neither saturated fatty acids nor amino acids or alcohols are oxidized, a study of the oxidations produced by gonococci is almost limited to a study of the breakdown of the glucose molecule. It will be seen in the course of this study that the oxidation of glucose by gonococci is incomplete and proceeds through a chain of reactions which we have been able to separate.

Methods

The problem of bacterial oxidations may be studied in two ways. The first and more general method is to study the chemical changes in a medium of known composition where active growth is taking place. The second method is to subject a given substrate to the action of relatively large amounts of bacteria under conditions under which growth does not occur because of the absence of nitrogenous constituents. The bacteria are termed "resting" or nonproliferating bacteria. Using such systems we may study the enzymic reactions of bacteria uncomplicated by the synthetic growth processes. This method, which was introduced by Quastel and his collaborators (5), has been employed by us.

The organisms were grown in an egg digest medium containing 1 per cent glucose and buffered at pH 7.4 (Miller and Castles (6)). This medium yields a plentiful growth of gonococcus. After 10 to 12 hours incubation the growth was washed off twice with 0.9 per cent NaCl and centrifuged. The organisms were suspended in 0.9 per cent NaCl containing 0.05 M phosphate buffer, usually at pH 7.38. The substrates whose oxidation was being tested were of the purest kind obtainable, and some of them were prepared in the laboratory. In the case of acids they were neutralized with NaOH.

The oxidations were performed in flat bottom Warburg vessels with the inner vessel containing the alkali suspended to the walls to insure free movement to the bacterial suspension and continuous saturation with oxygen. The temperature of the bath was 37°. Since the organisms themselves in the absence of any oxidizable material take up practically no oxygen, the thermobarometer contained the bacterial suspension without the substrate. In this manner the oxygen consumed by the bacteria in presence of a substrate represents the oxidation of the substrate alone. Extreme care was taken to avoid contamination, and vessels and pipettes were sterilized before use. All the figures given are the averages of at least fifteen experiments.

Oxidation of Carbohydrates by Gonococci

Gonococci possess quite a selective action in their oxidation of carbohydrates, as glucose is the only substance belonging to this group which is readily oxidized by the microorganism. The following hexoses have been tested: glucose, levulose, galactose, and mannose; among the pentoses, arabinose and xylose. As can be seen in Table I, glucose was the only sugar which was readily oxidized. The inability of gonococci to oxidize the other sugars has also been tested by substituting the other sugars for glucose in the culture medium and trying to grow the bacteria in such a medium. The microorganism failed to grow whenever glucose was absent from the culture medium. Cook and Stephenson (7) report that the oxidation of glucose by *Bacillus coli* is not carried to completion, stopping when about 4 molecules of oxygen are consumed per molecule of glucose. Gonococci, like *Bacillus coli*, fail to oxidize glucose completely. The oxygen uptake per molecule of glucose corresponds to the use of 2 molecules of oxygen per molecule of glucose, as can be seen in Fig. 1, which is taken from a series of twenty similar experiments. (In order to insure the completion of oxidation in a short time, a small amount of glucose was taken; namely, 0.005 millimol.) This would indicate that the final oxidation product is acetic acid, according to the following equation,

$$C_6H_{12}O_6 + 2O_2 = 2CH_3COOH + 2CO_2 + 2H_2O$$

Oxidation of Hydroxy Acids

A study of the oxidation of a variety of organic acids by gono-cocci gives further evidence of the simplicity of the oxidizing enzymes present in gonococci. We shall first consider the oxidation of hydroxy acids. It is well known that the introduction of an OH radical into a saturated fatty acid renders it more prone to oxidation by a variety of mild oxidants. The amount of energy required to oxidize a hydroxy acid is less when the OH radical occupies the α position. Gonococci oxidize only those hydroxy

TABLE I

Oxidation of Carbohydrates and of Hydroxy Acids by Gonococci

The bacteria have been suspended in 0.9 per cent NaCl and 0.05 M phosphate buffer, pH 7.38, temperature 37°.

Carbohydrate 0.03 mM in each vessel	O$_2$ consumption per hr. and per mg. dry bacteria	Hydroxy acid 0.03 mM in each vessel	O$_2$ consumption per hr. and per mg. dry bacteria
	c.mm.		c.mm.
Glucose...................	294.5	Lactic acid..............	337.2
Levulose..................	3.0	α-Hydroxybutyric acid...	215.0
Galactose.................	3.1	Glyceric acid...........	146.3
Mannose..................	3.8	Mandelic acid...........	37.3
Arabinose................	0	Glycolic acid...........	11.2
Xylose...................	0	Malic acid..............	7.9
		Gluconic acid...........	0
		d-Tartaric acid.........	0
		α-Hydroxyisobutyric acid..................	0
		β-Hydroxybutyric acid...	0

acids having the general formula R—CHOHCOOH. The following hydroxy acids belonging to this group have been tested: lactic acid, α-hydroxybutyric acid, glyceric acid, mandelic acid, glycolic acid, malic acid. The rate of oxidation of these acids decreases in the order named. It is interesting to point out that mandelic acid is very resistant to further oxidation in the animal body and only yields traces of benzoic acid when given to dogs (Dakin (8)). β-Hydroxybutyric acid, in which the OH radical is in the β position and α-hydroxyisobutyric acid, in which the hydrogen attached to
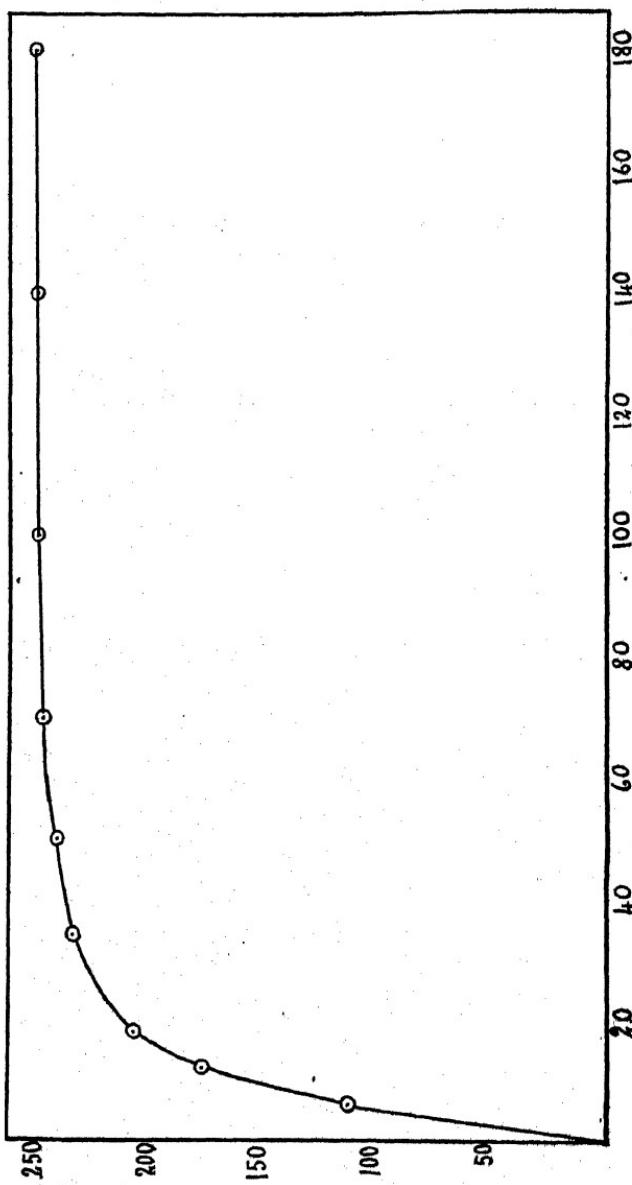
FIG. 1. The oxidation of glucose by gonococci. The abscissa measures time in minutes; the ordinate, oxygen consumption in c.mm. 0.005 millimol of glucose was used. The oxidation was performed at 37°. The calculated oxygen consumption for the oxidation of glucose to acetic acid was 254 c.mm.; found, 252 c.mm.

the carbon containing the OH radical is replaced by a non-polar group, are not oxidized by gonococci (Table I).

The oxidation of lactic acid by *Bacillus coli* ends when two-thirds of it have been oxidized (Cook and Stephenson). In the case of gonococci 1 molecule of lactic acid requires 1 molecule of oxygen, as can be seen in Fig. 2, where 0.01 millimol of lactic acid (as
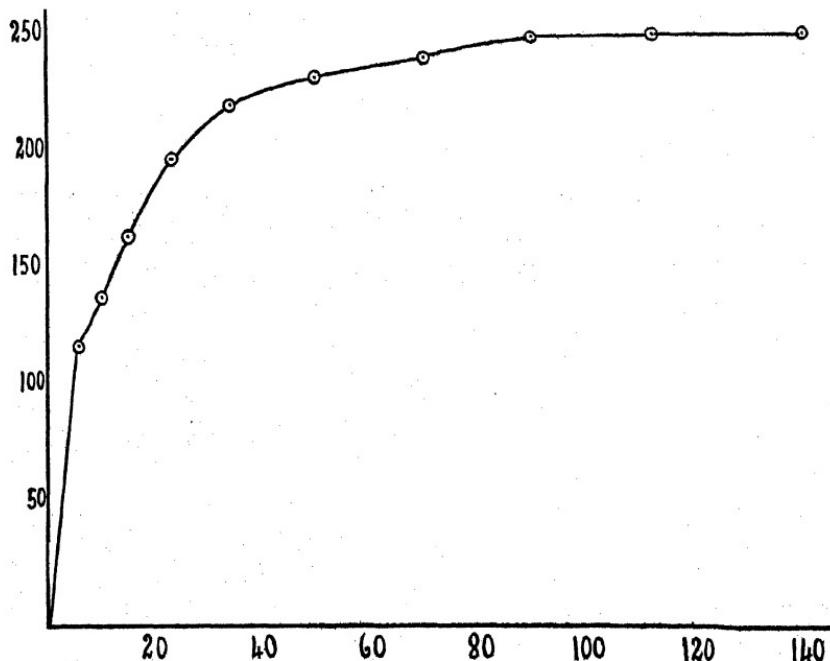
Fig. 2. The oxidation of lithium lactate by gonococci. The abscissa measures time in minutes; the ordinate, oxygen consumption in c.mm. 0.01 millimol of lactate was used. The oxidation was performed at 37°. The calculated oxygen consumption for the oxidation of lactic to acetic acid was 254 c.mm.; found, 251 c.mm.

lithium lactate twice recrystallized) was used. This would lead, as in the case of glucose, to acetic acid as end-product,

$$C_3H_6O_3 + O_2 = CH_3COOH + CO_2 + H_2O$$

If acetic acid is the end-product of the oxidation of lactic acid, there must be 1 mol of CO_2 formed for every mol of oxygen consumed. Such is the case, as can be seen in Table II in which are

tabulated the oxygen consumption and CO_2 production after complete oxidation of lithium lactate by the bacteria. The experiments were performed at pH 5.89. The amount of CO_2 dissolved in the suspension containing phosphates as buffer was calculated according to the data given by Van Slyke, Sendroy, Hastings, and Neill (9).

It is probable that the other α-hydroxy acids are oxidized in a similar manner to an acid of 1 carbon atom less and CO_2. Such is at least the case for α-hydroxybutyric acid which, like lactic

TABLE II

Oxidation of Lactate by Gonococci. Consumption of Oxygen and Production of CO_2

pH 5.89, temperature 37°, 0.01 mM of lithium lactate in each vessel. Calculated O_2 consumption, 254 c.mm.; CO_2 production 254 c.mm. for the oxidation of lactic acid to acetic acid.

Experiment No.	O₂ consumption	CO₂ production
	c.mm.	*c.mm.*
1	249.5	249.0
2	252.0	250.0
3	254.0	254.0
4	255.0	253.8
5	258.0	257.0
6	253.0	252.0
7	250.4	249.0
8	249.6	250.0
Average.............	252.7	251.8

acid, uses up 1 mol of oxygen and produces 1 mol of CO_2 for every mol of acid.

Oxidation of Ketonic Acids

α-Ketonic acids are highly reactive substances and therefore are oxidized with great ease by the usual laboratory oxidizing agents. Gonococci are also able to oxidize them. Pyruvic acid in its oxidation by gonococci uses 1 atom of oxygen (Fig. 3), which would lead to the same end-product already suggested, namely acetic acid, according to the equation,

$$CH_3COCOOH + \tfrac{1}{2}O_2 = CH_3COOH + CO_2$$
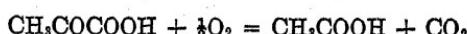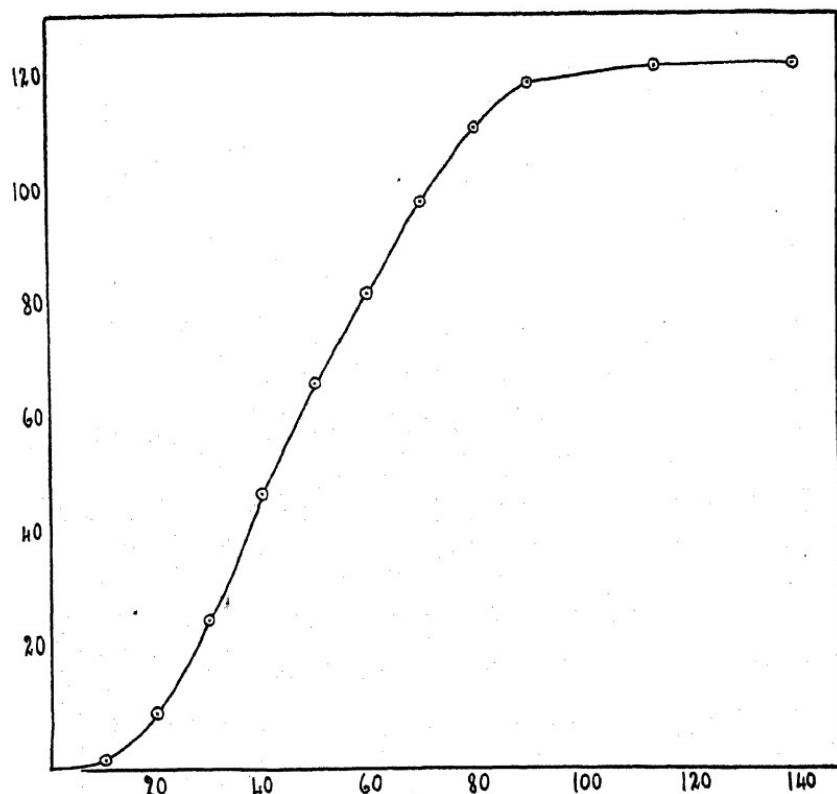
Fig. 3. The oxidation of sodium pyruvate by gonococci. The abscissa measures time in minutes; the ordinate, oxygen consumption in c.mm. 0.01 millimol of pyruvate was used. The oxidation was performed at 37°. The calculated oxygen consumption for the oxidation of pyruvic to acetic acid was 127 c.mm.; found, 122 c.mm.

TABLE III

Oxidation of Ketonic Acids by Gonococci

The bacteria have been suspended in 0.9 per cent NaCl and 0.05 M phosphate buffer, pH 7.38, temperature 37°.

Ketonic acid 0.03 mM in each vessel	O₂ consumption per hr. per mg. of dry bacteria
	c.mm.
Pyruvic acid..	113.0
Acetoacetic acid..	0
Levulinic acid..	0

It is interesting to observe that the curve of oxidation of pyruvic acid with its marked induction period resembles the curve which is obtained when plotting many autocatalytic reactions. The kinetics of this reaction will be discussed in another communication. (The pyruvic acid used in these experiments was always freshly distilled, at 47° and 2 mm. of Hg.) Neither acetoacetic acid, a β-ketonic acid, nor levulinic acid, a γ-ketonic acid, is oxidized by gonococci (Table III).

Organic Acids and Other Substrates Not Oxidized by Gonococci

The selective action of gonococci as regards its oxidizing power is more manifest when we take into consideration saturated fatty acids, some of which, *e.g.* formic acid, are oxidized to completion by *Bacillus coli*. The following acids which have been tested were not oxidized by gonococci: formic, acetic, propionic, butyric, succinic, oxalic, malonic, citric. In addition, we have tested the following amino acids, glycine, *d*-leucine, and *d*-alanine, and found that they were not oxidized by gonococci. Acetaldehyde, ethyl alcohol, and glycerol are not oxidized by gonococci. *Bacillus coli* (Quastel and Whetham (5); Cook and Stephenson), *Bacillus prodigiosus*, *Bacillus proteus* (Quastel and Wooldridge (10)) possess the ability to oxidize many of these acids. Even *Bacillus alkaligenes*, which according to Quastel and Wooldridge oxidized glucose only very slightly, is able to oxidize formic acid.

Breakdown of Glucose by Gonococci

It is the course of events which occur during the oxidation of glucose by gonococci which is of special biological interest. As Hopkins has pointed out (11) the accumulation of an end-product in metabolism is no measure of its importance, but depends on the relative rates of its production and removal. It is the highly reactive and elusive products which the cell utilizes for building up its own material, and it is for this reason that the fermentation chemist seldom catches sight of the biologically important products of fermentation. It is only by interfering with the normal course by the introduction of fixing agents for special products, as is done by Neuberg and his school, by the use of specific inhibiting

agents, or by altering the relative rates of production and removal that the presence of biologically important products can be demonstrated.

In most living cells (certain molds which oxidize glucose directly are an exception) the oxidation of glucose is performed via cleavage of the hexose. The breakdown of glucose by gonococci is effected in a similar manner, as we will presently show. The glucose molecule is broken down by the glycolytic enzyme into 2 molecules of lactic acid. The lactic acid is oxidized into pyruvic acid by the lactic acid-oxidizing enzyme, and finally the pyruvic acid is oxidized into acetic acid and CO_2.

Glycolytic Enzyme of Gonococci

The cleavage of glucose into lactic acid can be demonstrated by the use of agents which inhibit the oxidation of lactic acid and as a consequence lead to an accumulation of the acid, and by inhibition of the glycolytic enzyme which will prevent the oxidation of glucose. We have employed both methods to prove the existence of the glycolytic enzyme in gonococci. The oxidation of lactic acid can be inhibited either by submitting the bacteria to the absence of oxygen or by the use of KCN which, as is well known, inhibits cellular oxidations and has no effect on glycolysis. The lactic acid formed can be determined chemically or by the use of Warburg's manometric methods. We have used both methods.

Determination of Lactic Acid—A suspension of gonococci-containing glucose was shaken in a flask through which purified nitrogen was continuously bubbled. The temperature of the air bath where the incubation took place was 37°. As can be seen in Table IV, 0.44 millimol per liter of glucose disappeared at the end of 1 hour and 0.89 millimol of lactic acid was formed, which gives 2 molecules of lactic acid per molecule of glucose. The glucose was determined by the use of Folin's method (12) and the lactic acid by Friedemann's method (13). Identical results have been obtained by the use of Warburg's manometric methods (14), in which the lactic acid formed reacts with the sodium bicarbonate and gives up CO_2 stoichiometrically,

$$C_6H_{12}O_6 + 2CO_3HNa = 2CH_3CHOHCOONa + 2CO_2 + 2H_2O$$

The bacteria were suspended in Ringer's bicarbonate solution containing 0.5 per cent glucose, pH 7.4, and the vessels contained as gas phase 95 per cent nitrogen plus 5 per cent CO_2.

Sodium fluoride is a specific inhibitor of the glycolytic process of animal cells (Lipman (15), Dickens and Simer (16)). The same is true in the case of glycolysis by gonococci. 0.2 M NaF inhibits glycolysis completely, 0.02 M NaF produces 90 per cent inhibition, and 0.01 M, 51 per cent inhibition, as measured by the inhibition of glucose oxidation by the bacteria. Further proof that it is the cleavage of glucose into lactic acid which is responsible for the

TABLE IV

Glycolytic Enzyme of Gonococci $(C_6H_{12}O_6 \rightarrow 2C_3H_6O_3)$

	Production of lactic acid under anaerobic conditions. Gonococci suspension incubated for 1 hr. at 37°; purified N_2, bubbling continuously into vessels; 0.9 per cent NaCl + 0.05 M phosphate, pH 7.38		Production of CO_2 under anaerobiosis. Gonococci in Ringer's bicarbonate solution + 0.5 per cent glucose, pH 7.4; in 95 per cent N_2 + 5 per cent CO_2	
	Before incubation	After incubation	Experiment No.	CO_2 per hr. per mg. of dry bacteria
	*m*M *per l.*	*m*M *per l.*		*c.mm.*
Glucose.......................	5.44	5.0	1	26.2
Lactic acid...................	None	0.89	2	31.2
			3	26.9
			4	31.4
			5	27.5
			6	28.8

failure of glucose oxidation under fluoride action is given by the fact that NaF at 0.02 M and 0.01 M has no effect on the oxidation of lactic acid by gonococci (Fig. 4).

Much evidence has been accumulated during recent years by Neuberg and his collaborators in proof of the hypothesis that the glucose molecule during the process of fermentation by animal cells is converted first into the highly reactive methylglyoxal which subsequently changes into lactic acid. The ferment glyoxalase which changes methylglyoxal into lactic acid was discovered simultaneously by Dakin (17) and Neuberg (18). Neuberg and Gorr (19), working with a heavy suspension of *Bacillus coli*,

obtained a quantitative change of methylglyoxal to lactic acid and concluded that methylglyoxal may be the precursor of lactic acid in the breakdown of glucose.   It seems that with gonococci
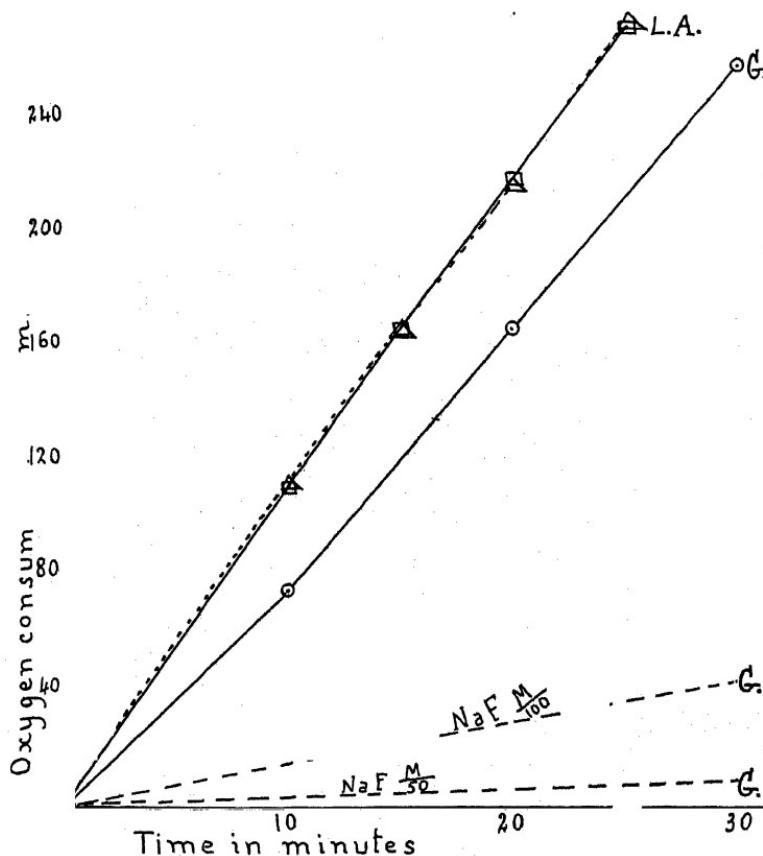
FIG. 4. The effect of sodium fluoride on the oxidation of glucose and lactic acid by gonococci.   G. with ⊙ on the solid line shows the oxidation of glucose; G., with the broken line, the oxidation of glucose in the presence of fluoride, 0.01 M and 0.02 M.   L. A., with □ on the solid line shows the oxidation of lactate; and with △ on the broken lines, the oxidation of lactate in the presence of fluoride, 0.02 M.

such is not the case.   If glucose in its cleavage into lactic acid had to pass through methylglyoxal, it is obvious that methylglyoxal would be as readily oxidized by gonococci as glucose.   But in fact

the oxidation of methylglyoxal was so slow as to be considered negligible, as can be seen in Fig. 5. In 2 hours 0.03 millimol of methylglyoxal consumed only 16 c.mm. of oxygen. At that time 0.03 millimol of lactic acid was added. There was a consumption of 108.7 c.mm. of oxygen in 15 minutes.

The methylglyoxal used in these experiments was prepared from dihydroxyacetone according to Fischer and Taube's method
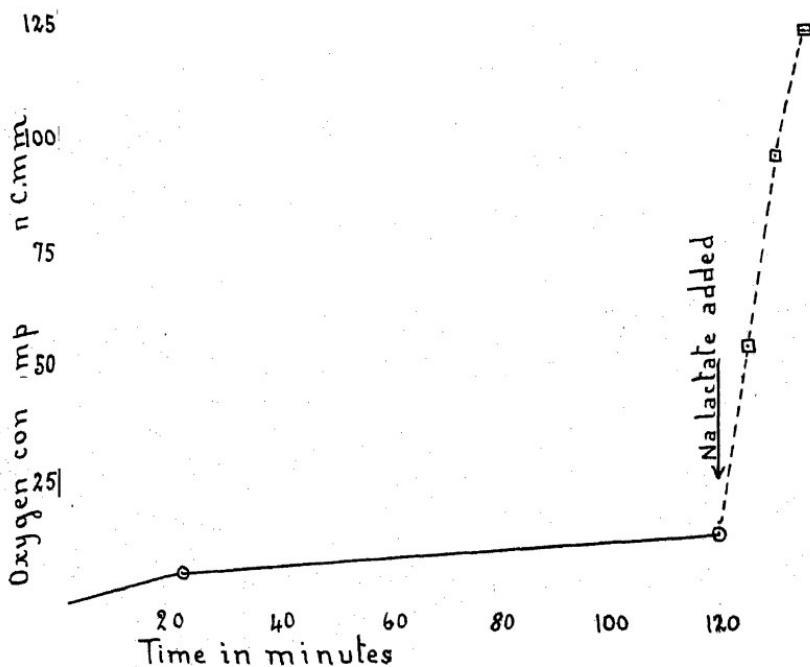
FIG. 5. The oxidation of methylglyoxal by gonococci. The small oxygen consumption by methylglyoxal (16 c.mm. in 2 hours) is contrasted with the rapid oxidation of lactate (108.7 c.mm. in 15 minutes).

(20). It was afterwards redistilled according to a method given by the same authors. The methylglyoxal was obtained as beautiful light yellow crystals which gave a colorless solution when dissolved in water and had all the properties mentioned by Fischer.[1]

It seems therefore probable that the glucose molecule when fermented by gonococci does not previously pass through methylglyoxal as is the case in many living cells.[2]

The glycolytic enzyme of gonococci is very susceptible to the effect of external agents and is destroyed with the death of the microorganism. The power of a gonococcus suspension to oxidize glucose can in fact be used as a test for the viability of the bacteria. As a consequence, the speed of glucose oxidation by a given suspension of bacteria decreases considerably, even in the same sample, on standing. Thus a gonococci suspension 10 hours old which oxidized glucose at the rate of 173 c.mm. per half hour, 3 hours after standing at room temperature oxidized at the rate of only 27 c.mm. per half hour, which represents an inhibition of 84 per cent. A gonococci suspension kept in the ice box for 24 hours has completely lost the power to oxidize glucose. That in such cases it is the glycolytic ferment which has been destroyed is demonstrated by the fact that lactic acid is readily oxidized in such circumstances, as we will presently show. This extreme susceptibility of the glycolytic enzyme of gonococci is to be contrasted with the relative resistance shown in the case of *Bacillus coli*. The English investigators report that bacterial suspensions of *Bacillus coli* kept in the ice box oxidize glucose readily.

The fermentative power of gonococci is extremely low with relation to its oxidative power. In effect, the Pasteur reaction as defined by Warburg (21), namely the excess fermentation given by the following formula, $U = QM^{N_2} - 2 \ (QO_2)$ (where QM^{N_2} is the anaerobic glycolysis and QO_2 the respiration), is highly negative as can be seen from the figures given below.

$Q_M^{N_2} = 28.6$ c.mm. (CO_2 produced per hour by 1 mg. of dry bacteria in presence of glucose and in N_2CO_2)

$QO_2 = 294.5$ c.mm. (oxygen consumed per hour by 1 mg. of dry bacteria in presence of glucose in atmospheric oxygen)

$U = -560.4$

This is in concordance with the highly aerobic character of the bacteria and the difficulty of growing them in liquid medium when diffusion of oxygen is not favored by continuous agitation.

[2] The possibility that the sample of methylglyoxal used in these experiments failed to be oxidized because it was "inactive" while the methylglyoxal produced by the bacteria during the catabolism of glucose is in

Lactic Acid-Oxidizing Enzyme

Once the glucose molecule has been broken down into lactic acid, the first step in the oxidation of this substance is pyruvic acid, a substance which is liable to escape observation owing to the ease with which it itself is broken down. The oxidation of lactic acid through pyruvic acid is performed also by *Bacillus coli*. Cook
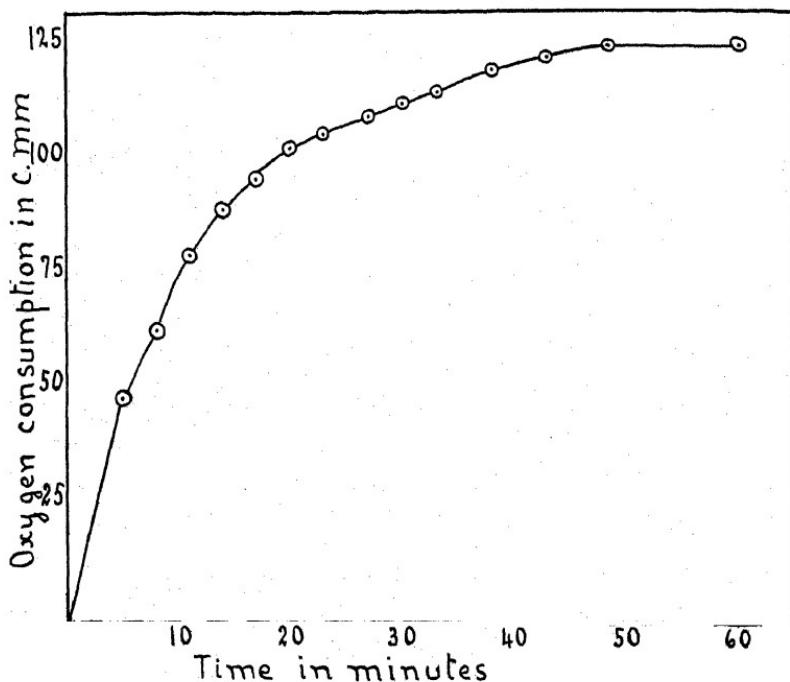
FIG. 6. The oxidation of lactate by gonococci kept in the ice box for 24 hours. 0.01 millimol of lithium lactate was used. The calculated amount of oxygen consumption for the oxidation of lactic acid to pyruvic acid was 127 c.mm.; found, 125 c.mm.

(22) has shown that pyruvic acid is produced in the aerobic oxidation of lactic acid by *Bacillus coli*, if the reaction is checked by the use of fixatives for pyruvic acid (sodium bisulfate). Quastel, Stephenson, and Whetham (23) made the same observation when they studied the oxidation of lactic acid in the presence of sodium nitrite.

The existence of an enzyme in gonococci concerned with the

oxidation of α-hydroxy acids to α-ketonic acids has been proved by the following methods.

(a) When a suspension of gonococci is kept in the ice box for 24 hours, this suspension readily oxidizes lactic acid and is unable to oxidize either glucose or pyruvic acid. The oxidation of lactic acid is ended when 1 molecule of lactic acid has consumed 1 atom of oxygen, which would be in accordance with the following equation (Fig. 6),

$$CH_3CHOHCOOH + \tfrac{1}{2}O_2 = CH_3COCOOH + H_2O$$
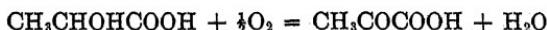
The determination of the pyruvic acid thus formed was performed by taking advantage of the property of dried yeast or yeast juice

TABLE V

Oxidation of Glucose, Lactic Acid, and Pyruvic Acid at pH 8.98 (Borate Buffer); Temperature, 37°

Time	O₂ consumption		
	Glucose	Na lactate	Na pyruvate
min.	*c.mm.*	*c.mm.*	*c.mm.*
6	0	28.0	0
12	0	48.1	0
18	0	72.8	0
24	0	95.0	0
30	0	112.0	0

to decarboxylate pyruvic acid by means of the enzyme decarboxylase discovered by Neuberg and Hildescheimer (24). These experiments will be reported in a coming paper.

(b) When the gonococci are suspended in a solution of pH 9.0 (borate buffer) neither glucose nor pyruvic acid is oxidized, while lactic acid is oxidized (Table V).

The lactic acid-oxidizing enzyme is very resistant to the effect of physical agents—bacterial suspensions of gonococci repeatedly frozen and thawed with the aid of liquid air and warm water (no disruption of cells is observed microscopically after such treatment) almost lost their power to oxidize glucose and pyruvic acid, while they readily oxidized lactic acid, as can be seen in the following figures. Bacteria were frozen and thawed repeatedly (liquid air and warm water), centrifuged, and suspended in 0.9 per cent

NaCl + 0.05 M phosphate buffer, pH 7.26. The O_2 consumption in c.mm. per half hour was 5.0 with glucose, 188.0 with lactic acid, and 9.0 with pyruvic acid.

Pyruvic Acid-Oxidizing Enzyme

The work of Neuberg and his colleagues gives great prominence to the part played by pyruvic acid in fermentation phenomena, and Aubel and Salabartan (25) have come to the conclusion that in the growth of *Bacillus coli* upon glucose, pyruvic acid serves as the source of the bacterial substrate. Quastel (26) arrives at similar conclusions. Since in our experiments the necessary conditions for synthetic reactions via pyruvic acid have been abolished because the bacteria are suspended in a solution deprived of nitrogenous products, the enzyme responsible for the oxidation of α-ketonic acids oxidizes pyruvic acid to the same final step as that postulated for the oxidation of glucose and lactic acid, namely, acetic acid, according to the following equation,

$$CH_3COCOOH + \tfrac{1}{2}O_2 = CH_3COOH + CO_2$$
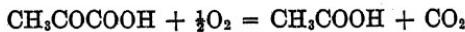
This would require the consumption of 1 atom of oxygen per molecule of pyruvic acid. As can be seen in Fig. 3, the amount of oxygen consumed by suspensions of gonococci in the presence of pyruvic acid corresponds to 1 atom per molecule, in agreement with the above equation.

Cook (22) maintains that *Bacillus coli* breaks pyruvic acid into a mixture of formic and acetic acids. Gonococci do not produce formic acid. We have incubated gonococci suspensions containing sodium pyruvate (5 cc. of 0.1 M sodium pyruvate) in flasks which were continuously being shaken to insure ample diffusion of oxygen. When incubation was ended the bacterial suspensions were acidified with sulfuric acid and distilled with steam in the presence of concentrated magnesium sulfate. The volatile acids were determined by direct titration of the distillate with 0.1 N sodium hydroxide with phenolphthalein as indicator. From 3.5 to 4.5 cc. of 0.1 N volatile acids were recovered. To detect the presence of formic acid in this distillate we alkalinized it with sodium carbonate and evaporated to a small volume in the water bath. Any pyruvic acid which could have passed during distillation was oxidized with hydrogen peroxide and the excess of this reagent

was destroyed with platinized platinum. No formic acid was detected in this residue.

Unlike the lactic acid-oxidizing enzyme, the pyruvic acid-oxidizing enzyme is extremely unstable and is promptly destroyed on standing. A bacterial suspension freshly prepared from cultures of 10 hours growth consumed 85 c.mm. of oxygen per half hour. A sample of the same suspension which was kept standing at room temperature ($\pm 25°$) for 4 hours, consumed only 22 c.mm. per half hour, which represents an inhibition of 74 per cent.

Comparative Rates of Oxidation of Glucose and Its Degradation Products

The speed of oxidation of these substrates decreases in this order: lactic acid > glucose > pyruvic acid (Fig. 7). The great speed with which lactic acid is oxidized is in marked contrast with the low speed with which pyruvic acid is oxidized. These differences in the velocity of reaction explain why the complete oxidation of lactic acid proceeds in two steps—first step, dehydrogenation, and second step, formation of a fatty acid of one C atom less and CO_2.

Influence of Hydrogen Ion Concentration

The influence of hydrogen ion concentration on the activity of oxidases has not been studied in such great detail as its influence on the activity of hydrolytic enzymes. In their studies of the pH activity of oxidases, Ohlson (27) (muscle succinoxidase), Quastel and Whetham (5) (succinoxidase of *Bacillus coli*), Dixon and Thurlow (28) (xanthineoxidase), and Bernheim (29) (potato aldehyde oxidase) have used the reduction of methylene blue as a test for the enzyme activity. The last author used also the reduction of nitrate. The pH activity of oxidases in the presence of molecular oxygen has received little attention. Hare (30) made an interesting study of the pH activity curve of tyramine oxidase in the presence of molecular oxygen. The optimum pH of this enzyme is about 10. Cook and Stephenson (7) studied the effect of pH on the oxidation of lactates by *Bacillus coli* and found the optimum to be around pH 5.6. Cook and Alcock (31) have recently studied the effect of pH on the oxidation of formates,

lactate, and succinate by the enzymes of *Bacillus coli*, using toluene-treated organisms.   The optimum pH found by these authors is 6 for the oxidation of formate, 6.3 for the oxidation of lactate, and 7.3 for succinate.
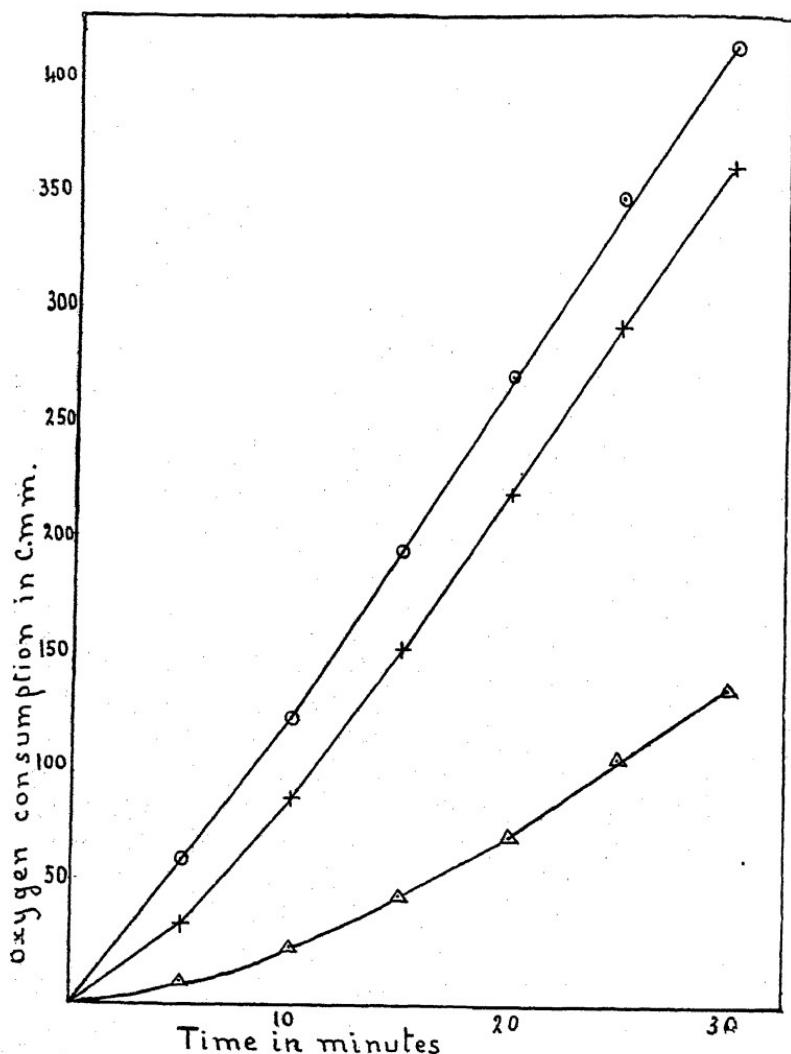
FIG. 7. Comparative rate of oxidation of glucose, lactic acid, and pyruvic acid by gonococci. Amount of substrates, 0.03 millimol. ⊙ indicates lactic acid; +, glucose; and △, pyruvic acid.

In our experiments we have suspended the bacteria (after washing and centrifuging) in the following buffers: acetate, citrate, phosphate, and borate.   The pH of the buffered bacterial suspension was determined electrometrically by the hydrogen electrode. The amount of the substrate was 0.03 mm.   The data indicate (Fig. 8) the oxygen consumption for 30 minutes.   The exchange of anions in the buffers of the same pH (phosphate and borate at pH 7.84; citrate and phosphate at pH 5.89) had no influence on the
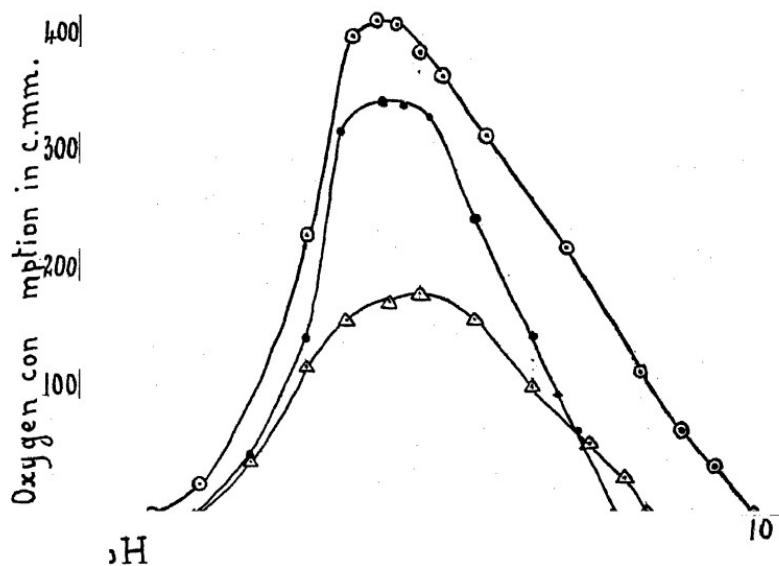
Fig. 8. The influence of pH on the oxidation of glucose, lactic acid, and pyruvic acid by gonococci. ● indicates glucose oxidation; ⊙, lactic acid oxidation; and △, pyruvic acid oxidation.

rate of oxidation of lactate by gonococci.   Michaelis (32) has introduced the veronal buffer which would be a useful buffer for working around pH 8 as the pK of diethylbarbituric acid is about 8.   Unfortunately veronal definitely retards the speed of oxidation of lactate by gonococci, probably because of its narcotic effect (Table VI).   As can be seen in Fig. 8, the optimum pH for the oxidation of glucose and lactate is about pH 6.7 and for the oxidation of pyruvate, about pH 7.0.   In each case there is a

narrow plateau around the optimum pH and from there the curves fall asymptotically on both sides.   On the acid side the oxidation of glucose and pyruvate ceases at pH 5 and the oxidation of lactate at pH 4.6.   On the alkaline side, the oxidation of glucose ceases at pH 8.7, the oxidation of pyruvate at pH 9, and that of lactate at pH 10.

TABLE VI

Influence of Anions of Buffers on Oxidation of Lactate by Gonococci
Amount of lactate 0.03 mM.

pH	Anion of buffer	O_2 consumption per 30 min.
		c.mm.
7.84	Phosphate	330.5
7.84	Borate	289.3
7.84	Diethylbarbiturate (veronal)	171.2
5.89	Phosphate	200.5
5.89	Citrate	202.0

DISCUSSION

The problem of biological oxidations in bacteria has made noteworthy progress during the last years, thanks to the introduction by Quastel of "resting" bacteria, where the microorganisms are kept in such a condition that proliferation is eliminated, so that they may be investigated in a manner similar to that used with enzyme or catalytic systems.   A growing organism with its complex synthetic machinery introduces factors, at present difficult to analyze, which may obscure the first changes that the metabolites undergo in the presence of the organism.

The extraordinary number of oxidations performed by *Bacillus coli* and the fact that even after successive washings of the bacteria the investigators were unable to suppress the so called endogenous respiration introduced a further complication, since besides the oxidation of known substrates there were oxidations of unknown substances which could not be controlled.   To find a bacterial cell able to perform only a small number of oxidations and devoid of endogenous respiration after the organism was washed and suspended in a buffered saline solution has been our first aim.   We have found such requirements in the gonococci.   The specificity

of the microorganism for the fermentation of glucose has allowed a study of the chain of reactions which proceed during the degradation of this molecule.

We have offered in this paper proof that the oxidation of glucose by gonococci proceeds in three steps: (1) fermentation of glucose into lactic acid, (2) oxidation of lactic acid into pyruvic acid, and (3) oxidation of pyruvic acid into acetic acid and CO_2 according to the following equations.

1. $C_6H_{12}O_6 \rightarrow 2\,C_3H_6O_3$ (fermentation)

2. $CH_3CHOHCOOH + \frac{1}{2}O_2 \rightarrow CH_3COCOOH + H_2O$ (1st step oxidation)

3. $CH_3COCOOH + \frac{1}{2}O_2 \rightarrow CH_3COOH + CO_2$ (2nd step oxidation)

Concerning the problem of the unity or plurality of the oxidizing enzymes, which has been and still is the subject of controversy between Thunberg's school (33) which maintains the specificity of the oxidizing enzymes and Warburg's view (34) that cellular oxidations are performed by a single enzyme (iron-containing), we offer evidence of the individuality of the enzymes concerned with the oxidation of α-hydroxy acids which perform dehydrogenations and the enzymes concerned with the oxidation of α-ketonic acids. We will call them for convenience, *α-hydroxyoxidase* and *α-ketonoxidase*. We have differentiated these two oxidizing enzymes simply by changing the hydrogen ion concentration of the medium or by letting the bacterial suspension stand in the ice box for 24 hours. While the α-ketonoxidase is destroyed at pH 9.0 and on standing, the α-hydroxyoxidase remains unaltered. But if there is a specificity of oxidizing enzymes, this is a group specificity and not individual specificity. In other words, all the acids belonging to the group of α-hydroxy acids are oxidized by the same enzyme. The different rates of their oxidation are due only to varying degrees of affinity between enzyme and substrate. In the same way the acids belonging to the group of α-ketonic acids are oxidized by a single enzyme. This will be shown in a coming publication.

We have studied the influence of hydrogen ion concentration on the activity of the enzymes concerned with the breakdown of the carbohydrate molecule by gonococci. The optimum pH for the oxidation of glucose and lactate is about pH 6.7 and for the oxida-

tion of pyruvate, about pH 7. As the dissociation constants of lactic acid and pyruvic acid are 1.38×10^{-4} and 5.6×10^{-3}, respectively, there appears to be some relation between the degree of dissociation of the acid concerned and the activity of the oxidase.

SUMMARY AND CONCLUSIONS

1. Gonococci possess a selective fermenting action upon glucose. Levulose, galactose, mannose, arabinose, and xylose are neither fermented nor oxidized by the bacterial cell.

2. The breakdown of the carbohydrate molecule by gonococci in presence of atmospheric oxygen and in the absence of nitrogenous constituents is performed in three steps: first, fermentation of glucose into lactic acid; second, oxidation of lactic acid to pyruvic acid; third, oxidation of pyruvic acid into acetic acid and CO_2. The oxidation of 1 molecule of glucose is ended when 2 molecules of oxygen are used up. The oxidation of lactic acid takes up 1 molecule of oxygen. The oxidation of pyruvic acid requires 1 atom of oxygen.

3. Lactic acid and pyruvic acid are oxidized by two separate enzymes which can be differentiated by performing the oxidation at pH 9 or by letting the bacterial suspension stand in the ice box for 24 hours. In such conditions only lactic acid is oxidized, the oxidation product being pyruvic acid.

4. α-Hydroxy acids and α-ketonic acids are also oxidized by gonococci. It is suggested that the enzyme concerned with the oxidation of α-hydroxy acids be named α-hydroxyoxidase and the enzyme concerned with the oxidation of α-ketonic acids, α-ketonoxidase. Neither saturated fatty acids nor amino acids are oxidized by gonococci.

5. The glycolytic enzyme and α-ketonoxidase are very unstable and lose their activity rather promptly once the bacteria are removed from the nutrient medium and kept in suspension in 0.9 per cent NaCl. The α-hydroxyoxidase is extremely resistant. A bacterial suspension can be kept in the ice box for 1 month and more without the α-hydroxyoxidase being destroyed.

6. The pH activity of the enzymes concerned with the breakdown of the glucose molecule has been studied. The optimum pH for the oxidation of glucose and lactate is about pH 6.7 and for the oxidation of pyruvate, pH 7. On the acid side the oxidation

of glucose and pyruvate ceases at pH 5 and that of lactate at pH 4.6. On the alkaline side the oxidation of glucose ceases at pH 8.7, the oxidation of pyruvate at pH 9, and that of lactate at pH 10.

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THE RELATION OF CHEMICAL STRUCTURE TO THE RATE OF HYDROLYSIS OF PEPTIDES

VII. HYDROLYSIS OF DIPEPTIDES BY ALKALI

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Great differences have been observed in the rates of hydrolysis of dipeptides as well as in those of ketopiperazines in alkaline medium.[1,2] Glycyl-glycine and glycyl-glycine anhydride, the simplest representatives of these two types of compounds, are much more rapidly hydrolyzed than any of their respective derivatives obtained by substitution of the hydrogen atoms of the methylene groups with alkyl or aryl groups.

Measurements, as effected on a series of ketopiperazines of the type represented in I, have shown[2] that the stability of the ring

$$R-\underset{\underset{H}{|}}{\overset{\overset{R'}{|}}{C}}\underset{NH}{\overset{|}{\underset{|}{\overset{|}{}}}}\cdots$$

R—C —— CO
$\,|\qquad\;|$
NH   NH
$\,|\qquad\;|$
CO——C—H
$\qquad\;|$
$\qquad\;$H
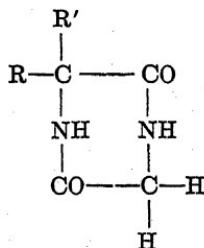
I

structure varies greatly with the nature of the radicles R and R' attached to the nucleus. The names of some of the anhydrides

[1] Levene, P. A., Bass, L. W., and Steiger, R. E., *J. Biol. Chem.*, **82**, 167 (1929).

[2] Levene, P. A., Rothen, A., Steiger, R. E., and Osaki, M., *J. Biol. Chem.*, **86**, 723 (1930).

studied, arranged in the order of decreasing rates of hydrolysis or
of increasing ring stability, are given in Table I.   In these experi-
ments 5 mols of sodium hydroxide had been allowed to act upon
1 mol of ketopiperazine, the initial concentration of sodium hy-
droxide being 0.5 N and the temperature 25°.

In an earlier paper[1] the hydrolysis of several dipeptides by alkali
had been studied.   The experimental conditions were the same as
those above.   Both the rates of hydrolysis of glycyl-alanine and

TABLE I

Ketopiperazines Arranged in Order of Increasing Ring Stability

Glycyl-glycine anhydride (symmetric)
Alanyl-glycine (or glycyl-alanine) anhydride
Leucyl-glycine (or glycyl-leucine) anhydride
Valyl-glycine (or glycyl-valine) anhydride
α-Aminoisobutyryl-glycine (or glycyl-α-aminoisobutyric acid) anhydride

TABLE II

Hydrolysis Constants of Peptides

Peptides, Series A	$k \cdot 10^3$	Peptides, Series B	$k \cdot 10^3$
Glycyl-glycine...............	152*	Glycyl-glycine.............	152*
Alanyl-glycine...............	32*	Glycyl-alanine.............	37*
Leucyl-glycine..............	7.3	Glycyl-leucine.............	9.3
Valyl-glycine................	0	Glycyl-valine..............	7.5
α-Aminoisobutyryl-glycine†		Glycyl-α-aminoisobutyric acid.....................	0*

* Figures previously reported.
† Not available for investigation.

of alanyl-glycine were found to be considerably lower than that
of glycyl-glycine.   Glycyl-α-aminoisobutyric acid was not hy-
drolyzed by the alkali of the concentration used.

The following dipeptides, d,l-leucyl-glycine, d,l-valyl-glycine,
glycyl-d,l-leucine, and glycyl-d,l-valine, have since been prepared
and submitted to the action of alkali under the conditions adopted
in all our previous work.   Their hydrolysis constants, together
with those of the peptides mentioned in the preceding paragraph,
are given in Table II.   Peptides of Series A are derived from

glycyl-glycine by substitution on the methylene group of the chain carrying the amino group (see II).   Peptides of Series B are
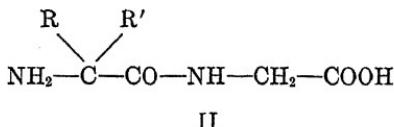
$$NH_2-\underset{\underset{\displaystyle II}{}}{\overset{\displaystyle R \diagdown \diagup R'}{C}}-CO-NH-CH_2-COOH$$

derived from glycyl-glycine by substitution on the methylene group of the chain carrying the carboxyl group (see III).   Members

$$NH_2-CH_2-CO-NH-\underset{\underset{\displaystyle III}{}}{\overset{\displaystyle R \diagdown \diagup R'}{C}}-COOH$$
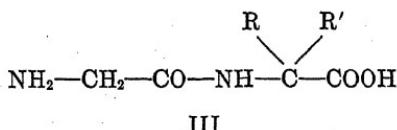
of each series are named in the order of decreasing rates of hydrolysis or increasing stability.

An interesting parallelism in the behavior towards alkali of dipeptides and of ketopiperazines becomes apparent from a comparison of the contents of Tables II and I: *any two radicles, R and R', have the same relative effect on the stability of the peptide linkage as on the stability of the ring structure.*

When classified according to increasing stabilizing effects, these radicles appear for all the series of compounds considered in the following order.

R	H	CH$_3$	CH$_2$CH$\big\langle{}^{CH_3}_{CH_3}$	CH$\big\langle{}^{CH_3}_{CH_3}$	CH$_3$
R'	H	H	H	H	CH$_3$
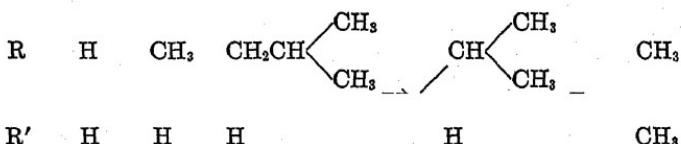
The experimental data are recorded in Table III.   This also contains hydrolysis figures obtained anew for the dipeptide alanyl-alanine, already studied some years ago.[1]   The titration values found at that time for this compound were somewhat irregular and it has since been felt that the experiment should be repeated.   As a result, the hydrolysis constant $k \cdot 10^3 = 6.5$ (previously found = 7.6) has been obtained.   Finally, figures are presented showing that the dipeptide leucyl-leucine is resistant to the action of the alkali of the concentration used

Thus, both glycyl-α-aminoisobutyric acid ($k \cdot 10^3 = 0$) and alanyl-alanine ($k \cdot 10^3 = 6.5$) are more stable than alanyl-glycine ($k \cdot 10^3 = 32$) or glycyl-alanine ($k \cdot 10^3 = 37$), while leucyl-leucine ($k \cdot 10^3 = 0$) is more stable than leucyl-glycine ($k \cdot 10^3 = 7.3$) or glycyl-leucine ($k \cdot 10^3 = 9.3$). It is seen from the preceding that *the stability of the peptide linkage is enhanced by the introduction in glycyl-glycine of a substituent R and still further, by the introduction of a second group R of the same nature.*

It can be seen from Table II that the rates of hydrolysis of the peptides of Series B are throughout higher than those of the corresponding isomers of the Series A. *If, into glycyl-glycine a group R be introduced, it stabilizes the peptide linkage to a greater extent when adjacent to the amino group than when adjacent to the carboxyl.*

The comparison of the hydrolysis constants of the two isomers glycyl-α-aminoisobutyric acid ($k \cdot 10^3 = 0$) and alanyl-alanine ($k \cdot 10^3 = 6.5$) shows that *two identical groups R, introduced into glycyl-glycine, stabilize the peptide linkage to a greater extent when attached to the same carbon atom than when distributed over both methylene groups of the chain.*

<div align="center">EXPERIMENTAL</div>

<div align="center">1. Preparation of Dipeptides</div>

<div align="center">d,l-Valyl-Glycine</div>

(a) *d,l-Bromoisovaleryl-Glycine*—This compound has already been obtained by Fischer and Schenkel.[3] Abderhalden, Sah, and Schwab[4] followed Fischer and Schenkel's indications. The procedure described has been modified as follows: A solution of 15 gm. (0.2 mol) of glycine in 100 cc. of 2.0 N sodium hydroxide (0.2 mol) was cooled in an ice-water mixture during treatment with 40 gm. (0.2 mol) of d,l-α-bromoisovaleryl chloride and 100 cc. of 2.0 N sodium hydroxide, these reagents being added in equivalent proportions with continuous shaking. The crude bromo compound precipitated as a solid on the addition of 52 cc. of 5.0 N hydrochloric acid (0.26 mol). It weighed dry, 42 gm. (yield, 88 per cent of the theory).

(b) *d,l-Valyl-Glycine*—This peptide was obtained for the first

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[3] Fischer, E., and Schenkel, J., *Ann. Chem.*, **354**, 13, 14 (1907).

[4] Abderhalden, E., Sah, P., and Schwab, E., *Fermentforschung*, **10**, 265 (1928–29).

time by Fischer and Schenkel.[3] These authors, having observed that the amination of the above bromo compound proceeded very slowly at room temperature, chose to aminate at 100°. The yield of crude product was 35 per cent of the theory. Secondary products, among which was an unsaturated one (presumably dimethylacrylyl-glycine), were formed in large amounts. Abderhalden, Sah, and Schwab[4] modified this procedure by aminating at 37° (4 days). Their yield was nearly the same as that of Fischer and Schenkel. They confirmed these authors' observations as to the formation of alcohol-soluble by-products. However, we have found that when the amination is carried out at room temperature in the way described below, the yield of dipeptide is the same as that already recorded and that the alcohol-soluble fraction then consists of the ammonium salt of unchanged d,l-bromoisovaleryl-glycine. 42 gm. of crude d,l-α-bromoisovaleryl-glycine were allowed to stand for 10 days at room temperature with 10 times this weight of ammonium hydroxide (sp. gr. 0.90). The solution was diluted with an equal volume of water and concentrated to dryness under reduced pressure. The crystalline residue was dissolved in 100 cc. of water and the solution was evaporated to dryness. This operation was repeated. The residue was then evaporated to dryness in the presence of 100 cc. of absolute alcohol. In order to remove the water completely, this was repeated four times. The final residue, a mixture of d,l-valyl-glycine and of the ammonium salt of unchanged d,l-α-bromoisovaleryl-glycine, was extracted with boiling absolute alcohol. The mixture was filtered while hot. The dipeptide which had remained undissolved was washed with hot absolute alcohol. It weighed 9 gm.

The alcoholic extract gave, on cooling, a crystalline precipitate of the *ammonium salt of d,l-α-bromoisovaleryl-glycine*. This was filtered off, washed with cold absolute alcohol, and air-dried. It weighed 13 gm. The filtrate was discarded. The suspension of this material in 35 cc. of water was treated with 14.5 cc. of 5.0 N hydrochloric acid, whereupon the free bromo acid precipitated. It was washed with water and air-dried. It weighed 10.9 gm. The *d,l-α-bromoisovaleryl-glycine* was crystallized from boiling benzene. It melted at 139–140° (uncorrected). Fischer and Schenkel[3] found 139–141° (corrected).

No. 190.   5.316 mg. substance: 6.914 mg. CO_2 and 2.405 mg. H_2O
          0.1000 gm.   "   : 4.33   cc. 0.1 N HCl (Kjeldahl)
          0.1168 "   "   : 0.0907 gm. AgBr (Carius)
$C_7H_{12}O_3NBr$ (238.03).   Calculated.   C 35.29, H 5.08, N 5.89, Br 33.58
                              Found.   " 35.46, " 5.06, " 6.06, " 33.04

The crude d,l-valyl-glycine was dissolved in about 30 cc. of water in the presence of some norit.   400 cc. of hot absolute alcohol were added to the filtrate.   Crystallization set in very soon; the mixture was cooled in ice and water.   5.6 gm. of dipeptide were obtained.   They were dissolved in 26 cc. of water in the presence of some norit; 400 cc. of hot absolute alcohol were added to the filtrate.   Crystallization took place immediately.   The mixture was allowed to stand overnight in ice and water.   The crystals were washed with alcohol.   Yield, 4.4 gm.

No. 198.   5.855 mg. substance: 10.420 mg. CO_2 and 4.490 mg. H_2O
        6.240 "   "   : 0.867 cc. N (23°, 757 mm.)
$C_7H_{14}O_3N_2$ (174.13).   Calculated.   C 48.24, H 8.10, N 16.09
                    Found.   " 48.53, " 8.58, " 15.96
                    Moisture, none

d,l-Leucyl-Glycine

(a) d,l-α-Bromoisocapronyl-Glycine—The preparation was carried out in a manner similar to that for d,l-α-bromoisovaleryl-glycine.

(b) d,l-Leucyl-Glycine[5]—49 gm. of crude d,l-α-bromoisocapronyl-glycine were allowed to stand for 3 days at room temperature with 10 times this weight of ammonium hydroxide (sp. gr. 0.90). The solution was then diluted with water, clarified with norit, filtered, and evaporated to dryness.   The residue was dissolved in about 16 parts of boiling water.   The solution was clarified with norit, then 1100 cc. of absolute alcohol were added.   Yield, 26 gm.   23.8 gm. of this material were dissolved in 16 parts of boiling water, 1100 cc. of absolute alcohol were added to the clear solution, and the mixture was left to stand in the cold room.   The peptide was washed with absolute alcohol.   It weighed dry, 19.6 gm.

No. 260.   5.195 mg. substance: 9.720 mg. CO_2 and 3.845 mg. H_2O
        0.1000 gm.   "   : 10.55 cc. 0.1 N HCl (Kjeldahl)
$C_8H_{16}O_3N_2$ (188.15).   Calculated.   C 51.03, H 8.57, N 14.89
                    Found.   " 51.04, " 8.28, " 14.77
                    Moisture, none

[5] Cf. Fischer, E., Ann. Chem., **340**, 144 (1905).

Glycyl-d,l-Valine

(a) *Chloroacetyl-d,l-Valine*—The details of the preparation of this compound were worked out 3 years ago. Meanwhile there appeared a paper by Abderhalden, Rindtorff, and Schmitz in which a method for preparing this compound is described.[6] The procedure given below is simpler. A solution of 23.5 gm. (0.2 mol) of d,l-valine in 100 cc. of 2.0 N sodium hydroxide (0.2 mol) was cooled in an ice-water mixture during treatment with 46 gm. (0.4 mol) of chloroacetyl chloride and 100 cc. of 5.0 N sodium hydroxide (0.5 mol), these reagents being added in equivalent proportions with continuous shaking. The chloroacetyl compound precipitated on addition of 72 cc. of 5.0 N hydrochloric acid (0.36 mol). It was washed with ice water, then with petrolic ether, and dried in a vacuum desiccator over phosphorus pentoxide and soda-lime. It weighed dry, 32 gm. (yield, 82 per cent of the theory).

(b) *Glycyl-d,l-Valine*—This compound was obtained in a somewhat different way from that described by Abderhalden, Rindtorff, and Schmitz.[6] Our procedure was as follows: A mixture of 32 gm. of crude chloroacetyl-d,l-valine with 10 times this weight of ammonium hydroxide (sp. gr. 0.90) was kept at room temperature for 3 days, during which time it was occasionally shaken. The solution was diluted with water, clarified with norit, and concentrated to dryness under reduced pressure. The residue was dissolved in 60 cc. of water. Norit was added. 700 cc. of absolute alcohol were added to the filtrate; the solution was allowed to cool in the ice box. Additional 300 cc. of alcohol were added to the mixture. The crystals were then filtered off and washed thoroughly with hot absolute alcohol. Yield, 23.5 gm. This product contained traces of chlorine and ammonia. It was dissolved in 70 cc. of hot water; norit and, after filtration, 500 cc. of absolute alcohol were added to the solution. The mixture obtained was cooled overnight in ice water. The crystals were washed with hot absolute alcohol and then dried in a desiccator over phosphorus pentoxide. Yield, 21 gm. As this material still contained 7.1 per cent of moisture, it was heated in a vacuum

[6] Abderhalden, E., Rindtorff, E., and Schmitz, A., *Fermentforschung*, **10**, 220 (1928–29).

oven to 70°.　For analysis the substance was redried at 100° under diminished pressure.

No. 182.　3.955 mg. substance: 6.990 mg. CO_2 and 2.845 mg. H_2O
　　　　　4.105 "　　　"　　: 0.583 cc. N (20°, 735 mm.)
　$C_7H_{14}O_3N_2$ (174.13).　Calculated.　C 48.24, H 8.10, N 16.09
　　　　　　　　　　　Found.　　" 48.19, " 8.05, " 16.00
　　　　　　　　　　　Moisture, 0.41 per cent

Glycyl-d,l-Leucine

(a) *Chloroacetyl-d,l-Leucine*—The preparation was carried out in a similar manner to that of chloroacetyl-d,l-valine.

(b) *Glycyl-d,l-Leucine*—Fischer[7] obtained this dipeptide by heating a mixture of 1 part of chloroacetyl-d,l-leucine and 5 parts of ammonium hydroxide to 100°.　This procedure was not followed.　Instead, the crude chloroacetyl-d,l-leucine was allowed to stand for 3 days at room temperature with 10 times its weight of ammonium hydroxide (sp. gr. 0.90).　The peptide formed was then isolated and purified in the usual way.

No. 261.　3.900　mg. substance: 7.330 mg. CO_2 and 3.105 mg. H_2O
　　　　　0.1000 gm.　　"　　: 10.48　cc. 0.1 N HCl (Kjeldahl)
　$C_8H_{16}O_3N_2$ (188.15).　Calculated.　C 51.03, H 8.57, N 14.89
　　　　　　　　　　　Found.　　" 51.25, " 8.90, " 14.67
　　　　　　　　　　　Moisture, none

Dextro-Alanyl-Dextro-Alanine

Sample 267 of this substance, described in a preceding publication,[8] was used.

Dextro-Leucyl-Dextro-Leucine

Sample 263 of this substance described in a preceding publication,[8] was used.

2. Procedure in Hydrolysis Experiments

5 mols of sodium hydroxide were allowed to act upon 1 mol of dipeptide, the concentration of sodium hydroxide being 0.5 N and the temperature 25°.　The procedure used for each peptide was the same as that described in a previous publication.[1]

[7] Fischer, E., *Ann. Chem.*, **340**, 157 (1905).
[8] Levene, P. A., Steiger, R. E., and Marker, R. E., *J. Biol. Chem.*, **93**, 605 (1931).

TABLE III

Hydrolysis Constants of Dipeptides*

d,l-Leucyl-glycine			d,l-Valyl-glycine			Glycyl-d,l-leucine			Glycyl-d,l-valine					Dextro-alanyl-dextro-alanine					Dextro-leucyl-dextro-leucine		
t	V (cc.)	$k \cdot 10^3$	t (days)	V (cc.)	$k \cdot 10^3$	t (days)	V (cc.)	$k \cdot 10^3$	t (days)	V (cc.)	t (days)	V (cc.)	$k \cdot 10^3$	t (days)	V (cc.)	t (days)	V (cc.)	$k \cdot 10^3$	t (days)	V (cc.)	$k \cdot 10$
08	2.54		0	2.53_5	0	0	2.53		0	2.53	0	2.51		0	2.53	0	2.52		0	2.54	0
	2.61	(11.4)	3	2.56_5	0	2	2.66	(11.6)	5.67	2.80	5.92	2.79	(8.8)	11	2.92	7.8	2.80	6.6	3	2.54	0
	2.78	7.3	6	2.56	0	4.17	2.75	9.6	10.83	2.96_5	7.92	2.86_5	(8.7)	15	3.03	13	3.00	6.7	9	2.58	0
	2.97	7.5	9	2.56_5	0	9.08	2.98_5	9.6	15.92	3.15	13.92	3.07	(8.4)	21	3.21	18	3.11	7.1	13	2.56_5	0
	3.19	7.3	13	2.55	0	10.96	3.07	9.6	22.88	3.33	20.92	3.28	7.7	28	3.36	23	3.26	6.5	18	2.58	0
	3.34	7.3	18	2.57	0	14	3.19	9.5	29.88	3.51	25.92	3.41	7.9	33	3.47	30	3.41	6.5	25	2.57	0
	3.47	7.2	25	2.57	0	17	3.30_5	9.5	33.88	3.59			7.8			37	3.57	6.6	32	2.58	0
	3.58_5	7.1	32	2.58	0	19	3.37	9.4					7.6					6.6			
	3.71	7.4				24	3.52	9.1					7.3					6.3			
						26	3.57	8.7					7.5					6.4			
						29	3.66	9.0					7.2					6.2			
						33	3.74	8.7					7.1					6.4			
Average		7.3			0			9.3					7.5					6.5			0

Values of $k \cdot 10^3$ in parentheses have not been included in calculating average values. $k \cdot 10^3$ is calculated for days.

The rates of hydrolysis were calculated with the following equation

$$k \cdot t = \log_{10} \frac{a}{a - x}$$

The results are given in Table III.

It may be said that not too great an importance should be attributed to the decimals given for $k \cdot 10^3$. The procedure used was not sufficiently precise to permit, for instance, of a definite statement to the effect that leucyl-glycine ($k \cdot 10^3 = 7.3$) is less rapidly hydrolyzed than glycyl-valine ($k \cdot 10^3 = 7.5$).

STROPHANTHIN

XXVII. RING III OF STROPHANTHIDIN AND RELATED AGLUCONES

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In previous work two of the rings of strophanthidin, and therefore of related aglucones, have been shown to be 6-membered.[1] More recently, new observations have been made which furnish evidence in regard to a third ring. On another occasion a monoanhydrodihydrostrophanthidin[2] was described, which was obtained by the action of acid on dihydrostrophanthidin. This substance has now become the important starting material for the series of substances which have given evidence as to the position and size of Ring III. It is formed by the loss of the tertiary hydroxyl group, OH^I, of dihydrostrophanthidin as water. Since dihydrostrophanthidin has the partial structure given in Formula I, the double bond of the monoanhydro derivative may have one of three positions (Formulæ II, III, and IV).

On oxidation of this substance in acetone solution with permanganate an acid, *monoanhydrodihydrostrophanthidinic acid*, of the formula $C_{23}H_{32}O_6$, was obtained, which resulted from the oxidation of the aldehyde group to carboxyl. If, however, the oxidation was performed in alkaline solution, a different acid was formed of the formula $C_{23}H_{34}O_8$, which resulted from the oxidation of the aldehyde group to carboxyl and the addition of two hydroxyl groups to the double bond. This acid readily formed a *methyl ester* which on acylation formed a *di-p-bromobenzoate*. On the basis of the locations assigned to the double bond in Formulæ II, III, and IV the new hydroxyl groups may have the positions given in Formulæ V, VI, and VII. When the ester was oxidized with

[1] Jacobs, W. A., and Gustus, E. L., *J. Biol. Chem.*, **74**, 805 (1927); **92**, 323 (1931).

[2] Jacobs, W. A., and Hoffmann, A., *J. Biol. Chem.*, **74**, 791 (1927).

chromic acid, an acid was formed which on analysis proved to have the formula $C_{24}H_{32}O_9$. Titration showed that the substance is a monobasic acid and it readily neutralizes a second equivalent of alkali on saponification, due to the original lactone group still present. Only a fraction of a third equivalent was consumed under the conditions employed, due to the partial saponification of the resistant methyl ester group. Further work showed that this acid was produced by the rupture of a ring between a secondary and a tertiary hydroxyl group with the formation of a carboxyl and a carbonyl group. Formulæ II and V are therefore excluded from further consideration.

Simultaneously, the original secondary hydroxyl group of dihydrostrophanthidin (OH^{III}) was also oxidized to the carbonyl. The acid is therefore a monomethyl ester of a dibasic diketo hydroxylactone acid, the *dimethyl ester* of which was readily prepared with diazomethane. For the former, any one of Formulæ VIII, IX, and X might be considered. In order to make a decision from among these, it was hoped to show the retention in this substance of the original OH^{II} of dihydrostrophanthidin by its behavior towards acids. If present, OH^{II} would be β to the keto group formed from OH^{III} and therefore form an unsaturated anhydro ketone, as in the case of the formation of anhydroisostrophanthonic acid from isostrophanthonic acid. This would at once eliminate Formula X. Both the acid and the ester were easily altered by acids but the reaction products were unworkable resins. In attempts also to prepare carbonyl derivatives no crystalline reaction products could be isolated.

The desired evidence was furnished by hydrogenation. Catalytic hydrogenation of the diketo acid was accomplished in ethyl acetate solution with the catalyst of Adams and Shriner. The reaction proceeded very slowly. The main product was neutral and on analysis gave figures in agreement with the formula $C_{24}H_{34}O_8$. A relatively small acid fraction was also formed. On attempting, however, to isolate the free acid, it was found to lactonize readily with the formation of a neutral substance which is apparently isomeric with the main neutral reaction product. In the formation of the latter it was obvious that the two carbonyl groups had been reduced to hydroxyl groups on one of which the carboxyl group lactonized with loss of water. It gave only a

monoacetate. The substance must be a *dihydroxydilactone ester.* This was confirmed by its behavior towards alkali. The ester group still exhibited a relative resistance towards alkali. On reacidification, the two saponified lactone groups readily closed again with the reformation of the dilactone ester. This behavior at once restricts the relative position of the carboxyl and carbonyl groups from which the new lactone group is formed to γ or δ. Formula X is, therefore, eliminated and the dilactone ester can have only Formula XI or XII.

The decision between these possibilities depended upon the determination of the identities of the groups involved in the new lactone group. This was established as follows: On reoxidation, a *hydroxyketodilactone ester,* $C_{24}H_{32}O_8$ (Formula XIII), was formed, which was further characterized by its *oxime.* The probability that the carbonyl group was formed from OH^{III} and that OH^{II} of the original strophanthidin molecule persisted through the whole series of reactions was supported by the ready formation of an *anhydroketodilactone ester,* $C_{24}H_{30}O_7$ (Formula XIV), by the action of dilute acid. The above dihydroxydilactone ester is stable under the same conditions. Formula XI, and not XII, therefore, represents this dihydroxydilactone ester. The formation of a *neutral sulfite* (Formula XV) by the action of thionyl chloride is a confirmation of this conclusion.[3]

It appears most likely that the new lactone group is δ and not γ. The former has its origin in the cleavage of a pentacyclic and the latter in that of a less probable tetracyclic system. We shall, therefore, provisionally accept from the present evidence that Ring III of strophanthidin and related aglucones is pentacyclic. Its position as given is apparently definitely established. It is joined to Ring I at carbon atoms (5) and (6) (Formula XVI). Whether it is substituted or not is a conclusion which must await more data. From certain reactions there is a suggestion that a methyl group is attached to Ring III on carbon atom number (17). It is not impossible that Ring IV will be either 5- or 6-membered and joined to carbon atoms (14) and (15) of Ring II. A provisional incompleted formula for strophanthidin might be represented as in Formula XVI.

[3] Jacobs, W. A., Elderfield, R. C., Grave, T. B., and Wignall, E. W., *J. Biol. Chem.*, **91**, 623 (1931).

                    CHO   OH^III
                    CH   C
                                     H
CH₂   CH——CH   CH   CH₂
CO   CH₂   COH^I   COH^II   CH···
     O   CH₂   CH   CH····

I   II

                    CHO   OH
                    CH   C
                                     H
—C   CH
            C   COH
        CH₂   CH·····

II

        CHO   OH
        CH   C
                     H
—CH   CH
  C   COH
CH₂   C·····

III

        CHO   OH
        CH   C
                     H
—CH   CH
  C   COH
CH   CH·····

IV

        COOH   OH
        CH   C
                     H
—COH   CH
  COH   COH
CH₂   CH·····

V

        COOH   OH
        CH   C
                     H
—CH   CH
  COH   COH
CH₂   COH·····

VI

    COOH   OH
    CH   C
—CH   CH
  COH   COH
HCOH   CH

VII

        COOR
        CH   CO
—CH   CH
    CO   COH
COOH CH

VIII

CH CO   CH CO   CH   C
                                               H
−CH CH   −CH CH   −CH   CH
CO COH   COH CO   CH   COH
                              O
CH₂ COOH   CH₂ COOH   CO   CH
                            −CH   CH−

IX   X   XI

COOR   COOR
CH   CH   CH   CO
              O
−CH   CH   CO   −CH   CH
              COH   CH   COH
HCOH   O
CH₂−   CO   CH
                     −CH   CH−

XII   XIII

COOR   COOR   COOR   H
CH CO   C   CO   CH   C
                                                  O
−C C   or   −C   C   −CH   CH
C   C   C   C   S=O
                                                  O
C   C   CH   C
                              O
                             CO   CH
                            −CH   CH−

XIV   XV

$$C^1HO$$
$$|\qquad\qquad OH$$
$$C^2H\qquad\quad C^{12}$$
$$\qquad\qquad\quad H$$

$$C^9H_2\text{——}C^8\text{———}C^7H\qquad C^3H\qquad C^{13}H_2$$
$$|\qquad\ \parallel\qquad\quad |\qquad\qquad |\qquad\qquad |$$
$$C^{10}O\qquad C^{11}H\qquad C^6OH\qquad C^4OH\qquad C^{14}H$$

$$O\qquad C^{18}H_2\qquad C^5H\qquad C^{15}H\ \cdot\quad CH$$
$$\qquad\qquad |\qquad\qquad |\qquad\qquad |$$
$$H_3C\text{—}C^{17}H\text{———}C^{16}H\qquad C\qquad\quad C$$

XVI

EXPERIMENTAL

Monoanhydrodihydrostrophanthidinic Acid, $C_{23}H_{32}O_6$—1 gm. of monoanhydrodihydrostrophanthidin was dissolved in 100 cc. of dry acetone. 0.9 gm. of finely powdered $KMnO_4$ was added and the mixture was stirred mechanically. Oxidation occurred promptly. After 2 hours the MnO_2 was filtered off and thoroughly extracted with water. The aqueous extract was acidified to Congo red with HCl, and the acid which precipitated was collected. Upon recrystallization by careful dilution of its acetone solution it formed irregular platelets and melted at 185–186° with decomposition. The substance was soluble in dilute Na_2CO_3 solution.

$[\alpha]_D^{24} = +72°$ (c = 0.800 in pyridine)
4.847 mg. substance: 3.355 mg. H_2O, 12.045 mg. CO_2
$\qquad\quad C_{23}H_{32}O_6$. Calculated. C 68.27, H 7.98
$\qquad\qquad\qquad\quad$ Found. " 67.77, " 7.74

The same substance was also obtained, although in poorer yield, if the oxidation was carried out in dry pyridine.

Tetrahydroxylactone Acid, $C_{23}H_{34}O_8$ *(Formula VII)*—20 gm. of monoanhydrodihydrostrophanthidin were dissolved in 150 cc. of pyridine and 800 cc. of 0.1 N NaOH were slowly added while the mixture was stirred mechanically. After 30 minutes, saponification of the lactone was complete. If the alkali is added at once, reprecipitation occurs and a longer time is required for resolution. 350 cc. of 5 per cent $KMnO_4$ solution were then added during 2 hours, with the temperature kept below 20°. The filtrate from

the MnO_2 was acidified to litmus with acetic acid and then concentrated in a vacuum to 300 cc. It was then acidified to Congo red with HCl. On standing, the acid deposited as long, silky needles. The yield of crude acid was 11 gm. After recrystallization from dilute acetone, it melted at 215–218° with decomposition after preliminary softening at about 160°. The melting point varied considerably.

4.122 mg. substance: 2.915 mg. H_2O, 9.555 mg. CO_2
$C_{23}H_{34}O_8$. Calculated. C 62.98, H 7.82
Found.   " 63.22, " 7.91

13.840 mg. of substance were titrated directly against phenolphthalein with 0.1 N NaOH. Calculated for 1 equivalent, 0.317 cc.; found, 0.306 cc. 3 cc. of 0.1 N NaOH were then added and the mixture was refluxed for 4 hours and then titrated back against phenolphthalein. 0.350 cc. of additional 0.1 N NaOH were consumed.

Methyl Ester of Tetrahydroxylactone Acid—The above acid was esterified in acetone solution with diazomethane. The ester is very readily soluble in all the usual organic solvents except petrolic ether. It crystallizes from ether as aggregates of platelets which melt at 207–208°.

$[\alpha]_D^{23} = +82°$ (c = 0.920 in pyridine)
4.315 mg. substance: 3.115 mg. H_2O, 10.110 mg. CO_2
3.970 "   "   : 2.225 " AgI
$C_{24}H_{36}O_8$. Calculated. C 63.68, H 8.02, OCH_3 6.86
Found.   " 63.90, " 8.08,   "   7.40

Di-p-Bromobenzoate of the Methyl Ester, $C_{24}H_{36}O_8$—The above ester was acylated with p-bromobenzoyl chloride in pyridine solution. The di-p-bromobenzoate is very sparingly soluble in methyl alcohol from which it crystallizes as long, rectangular plates which melt at 297–298°.

4.350 mg. substance: 2.060 mg. H_2O, 8.920 mg. CO_2
8.690 "   "   : 4.210 " AgBr
$C_{38}H_{42}O_{10}Br_2$. Calculated. C 55.72, H 5.19, Br 19.53
Found.   " 55.92, " 5.30,   " 20.62

Diketo Hydroxylactone Dibasic Acid Monomethyl Ester, $C_{24}H_{32}O_9$
(*Formula VIII*)—40 gm. of the above methyl ester, $C_{24}H_{36}O_8$, were

dissolved in 300 cc. of 90 per cent acetic acid and, while stirring mechanically, 175 cc. of Kiliani's CrO_3 solution were added slowly. The temperature was kept at 10–15°. After 1 hour, the mixture was diluted and $(NH_4)_2SO_4$ was added. On standing, the diketo acid separated as fine, stout prisms. The collected material was dissolved in dilute Na_2CO_3 solution. The solution was extracted several times with chloroform, which removed a small quantity of neutral impurities. On acidification of the carbonate solution with acetic acid, the acid was slowly reprecipitated. The substance is characterized by its extreme insolubility in the usual solvents. It can be recrystallized from a very large volume of acetone, from which it separates as micro boats which melt at 220–221° with decomposition.

$[\alpha]_D^{20} = +21°$ (c = 0.656 in pyridine)

3.900 mg. substance: 2.460 mg. H_2O, 8.882 mg. CO_2
3.850 "   "   : 1.950 " AgI
$C_{24}H_{32}O_9$. Calculated. C 62.04, H 6.95, OCH_3 6.68
                Found.   " 62.11, " 7.06,   "   6.69

15.714 mg. of substance were suspended in 1 cc. of alcohol and titrated against phenolphthalein with 0.1 N NaOH. Calculated for 1 equivalent, 0.339 cc.; found, 0.393 cc. 3 cc. of 0.1 N NaOH were then added and the mixture was refluxed for 4 hours and then titrated back. An additional 0.460 cc. of 0.1 N NaOH were used. During this treatment the resistant methyl ester was only partially saponified.

Dimethyl Ester of the Diketo Acid, $C_{24}H_{32}O_9$—The above acid was esterified in acetone suspension with diazomethane. The ester is easily soluble in the usual solvents. Upon recrystallization by careful dilution of its methyl alcoholic solution it forms flat prisms which soften to a mass of bubbles at 116–118° and then melt at about 180°. For analysis it was dried at 80° and 20 mm.

4.050 mg. substance: 2.660 mg. H_2O, 9.275 mg. CO_2
4.353 "   "   : 4.275 " AgI
$C_{25}H_{34}O_9$. Calculated. C 62.73, H 7.16, OCH_3 12.97
                Found.   " 62.46, " 7.35,   "   12.98

Dihydroxydilactone Ester, $C_{24}H_{34}O_8$ (Formula XI)—The catalytic reduction of the above diketo acid, $C_{24}H_{32}O_9$, goes on with extreme difficulty. 1 gm. of the acid was finely ground and suspended in a

large volume (about 500 cc.) of ethyl acetate. After shaking for several days in an atmosphere of purified hydrogen with 0.5 gm. of Adams and Shriner catalyst, 2 mols of hydrogen were absorbed. During this operation the sparingly soluble starting material gradually dissolved. Complete solution was taken as the sign of completed reaction. The filtrate from the catalyst was washed with Na_2CO_3 solution, which removed the isomer to be described below. The ethyl acetate solution was concentrated to a small volume and chilled. The dilactone separated as fine needles. However, it appeared to be contaminated with a small amount of a persistent impurity which repeated recrystallizations from a variety of solvents did not remove and which caused the carbon figures to come slightly high. This was observed with repeated preparations. In subsequent derivatives, however, this discrepancy was corrected. The substance is sparingly soluble in the alcohols, chloroform, acetone, and ethyl acetate. It melts at 280–281°.

$[\alpha]_D^{27} = +27°$ (c = 0.940 in pyridine)
4.452 mg. substance: 3.090 mg. H_2O, 10.760 mg. CO_2
4.435 "   "   : 2.968 "   "   10.482 "   "
$C_{24}H_{34}O_8$.   Calculated.   C 63.96, H 7.61
                    Found.   " 64.47, " 7.60
                      "   " 64.46, " 7.49

14.592 mg. of substance were suspended in 1 cc. of alcohol and 3 cc. of 0.1 N NaOH and the mixture was refluxed for 2 hours and then titrated back against phenolphthalein. Calculated for 2 equivlents, 0.648 cc.; found, 0.682 cc. The resistant methyl ester was only partly saponified. Upon acidification of the saponification mixture, the original dilactone was recovered.

1.880 mg. substance: 1.285 mg. H_2O, 4.425 mg. CO_2
Found.   C 64.19, H 7.65

The Na_2CO_3 extract of the ethyl acetate solution was acidified with acetic acid and concentrated in a vacuum at room temperature to a small volume. Stout rectangular prisms of a neutral substance deposited, which were collected. In contrast to the previous substance it is very soluble in acetone and crystallizes on dilution as fine needles. It decomposes to a solid at 243–244° which melts at 281–282°.

$[\alpha]_D^{22} = +34°$ (c = 0.890 in pyridine)
4.226 mg. substance: 2.830 mg. H_2O, 9.940 mg. CO_2.
Found.   C 64.15, H 7.49

Acetate of the Dihydroxydilactone Ester, $C_{24}H_{34}O_8$—0.1 gm. of the isomer melting at 280–281° was refluxed in 5 cc. of acetic anhydride for 30 minutes.   The solution was diluted, and, after decomposition of the acetic anhydride, was extracted with chloroform.   The chloroform solution was washed free from acid and concentrated to dryness.   The residue was recrystallized by careful dilution of its acetone solution.   The acetate formed stout prisms and melted at 216–217°.

4.766 mg. substance: 3.145 mg. H_2O, 11.025 mg. CO_2
$C_{26}H_{36}O_9$.   Calculated.   C 63.38, H 7.37
Found.   " 63.09, " 7.38

Sulfite of the Dihydroxydilactone Ester, $C_{24}H_{34}O_8$—0.1 gm. of the isomer melting at 280–281° was simultaneously chilled and covered with 2 cc. of thionyl chloride.   Immediate solution occurred with effervescence.   After standing 45 minutes, the excess reagent was removed in a vacuum.   The residue crystallized as rhombs under methyl alcohol.   After recrystallization from methyl alcohol, in which it is sparingly soluble, the substance melts at 229° with decomposition.

4.495 mg. substance: 2.650 mg. H_2O, 9.640 mg. CO_2
10.276 "   "   : 4.590 " $BaSO_4$
$C_{24}H_{32}O_9S$.   Calculated.   C 58.02, H 6.51, S 6.47
Found.   " 58.49, " 6.58, " 6.28

Hydroxyketodilactone Ester, $C_{24}H_{32}O_8$ *(Formula XIII)*—0.4 gm. of the above dilactone ($[\alpha] = +27°$) was dissolved in 10 cc. of 90 per cent acetic acid and oxidized with 2 cc. of 20 per cent CrO_3 solution. Kiliani's reagent, which contains sulfuric acid, could not be used since it caused dehydration in the process of extracting the product. After 30 minutes the reaction mixture was diluted and after warming the mixture was extracted repeatedly with hot chloroform.   The chloroform extract was washed free of acetic acid with hot water and concentrated to dryness.   The residue was recrystallized from acetone and formed fine rectangular prisms which melted at 159–160° with decomposition.

$[\alpha]_D^{27} = +21°$ (c = 0.640 in pyridine)

4.382 mg. substance: 2.895 mg. H_2O, 10.290 mg. CO_2

$\qquad$ $C_{24}H_{32}O_8$.   Calculated.   C 64.25, H 7.19

$\qquad\qquad\qquad$ Found.   " 64.04, " 7.39

Oxime of the Hydroxyketodilactone Ester, $C_{24}H_{32}O_8$—The oxime was prepared by refluxing the ketone with twice the theoretical quantities of hydroxylamine hydrochloride and sodium acetate in alcohol for 5 hours.   It crystallizes from dilute methyl alcohol as irregular platelets which melt at 195–200° after softening at about 175°.

$\qquad$ 6.175 mg. substance: 0.158 cc. N_2 (27°, 747.3 mm.)

$\qquad\qquad$ $C_{24}H_{33}O_8N$.   Calculated, N 3.02; found, N 2.87

Anhydroketodilactone Ester, $C_{24}H_{30}O_7$ *(Formula XIV)*—0.15 gm. of the ketodilactone, $C_{24}H_{32}O_8$, was refluxed in 10 cc. of methyl alcohol and 0.5 cc. of HCl (1.19) for 15 minutes.   The solution was then boiled down to 2 to 3 cc., and on chilling the anhydro ketone crystallized as hexagonal leaflets.   After recrystallization from methyl alcohol it melted at 198°.

$[\alpha]_D^{28} = +68°$ (c = 0.880 in pyridine)

4.427 mg. substance: 2.830 mg. H_2O, 10.805 mg. CO_2

$\qquad$ $C_{24}H_{30}O_7$.   Calculated.   C 66.94, H 7.03

$\qquad\qquad\qquad$ Found.   " 66.56, " 7.15

When the dilactone, $C_{24}H_{34}O_8$, was boiled for 30 minutes with methyl alcoholic HCl and concentrated as in the process used above, it was recovered unchanged.

$\qquad$ 4.428 mg. substance: 3.050 mg. H_2O, 10.440 mg. CO_2

$\qquad\qquad$ Found.   C 64.30, H 7.71

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THE ERGOT ALKALOIDS

I. THE OXIDATION OF ERGOTININE

By WALTER A. JACOBS

(From the Laboratories of The Rockefeller Institute for Medical Research,
New York)

Some time ago we became interested in the problem of the structure of the ergot alkaloids. Experiments on the oxidative degradation of ergotinine were already in progress when Barger's monograph[1] came to our attention, in which certain observations of Soltys[2] were discussed. The latter have since been separately published. In this work benzoic acid was obtained on oxidation of each of the alkaloids with permanganate. When nitric acid was used as the oxidizing agent, p-nitrobenzoic acid was the only substance recovered.

In our own experiments we have also used nitric acid and regularly obtained p-nitrobenzoic acid in addition to small amounts of benzoic acid. Since, however, we have constantly isolated from the reaction mixture a third crystalline substance of more complicated character, we wish to present a preliminary report of these experiments. After oxidation of the alkaloid as later described, benzoic acid, p-nitrobenzoic acid, and other substances were removed by ether extraction. The remaining aqueous solution yielded a sparingly soluble, ether-insoluble, substance in a naturally poor yield (6 per cent). The first suspicion that the substance is a nitro derivative was removed by the negative outcome of reduction experiments. It was then found to have retained the methyl-imino group of the parent ergotinine, the only one of the original 5 nitrogen atoms which remained. The analytical figures indicated a formula $C_{14}H_9O_8N$. Repeated titrations of a number of preparations were in excellent agreement for a substance containing three carboxyl groups. The remaining 2 oxygen atoms have

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[1] Barger, G., Ergot and ergotism, London (1931).
[2] Soltys, A., Ber. chem. Ges., **65**, 553 (1932).

not yet been accounted for. No evidence of the presence of a lactone group could be obtained on boiling with alkali. A methyl indole or perhaps a methyl hydroquinoline nucleus appears to be indicated in this substance. Whether a third, perhaps furane, ring is present cannot be considered until further data are available.

Attempts to prepare a methyl ester with diazomethane resulted only in a non-crystalline reaction product. When the tribasic acid was heated with methyl alcoholic hydrochloric acid, only one carboxyl group was esterified with the formation of a *mono-methyl ester*. The acid is readily oxidized by permanganate. Owing to the difficulty of procuring sufficient material, a report of this reaction will be left to a later communication.

Further work on the oxidative degradation of the ergot alkaloids is in progress.

<div align="center">EXPERIMENTAL</div>

In the oxidation of ergotinine with nitric acid a number of experiments was made in which the procedure was modified some-what in each case according to the objective. The following experiments are reported as they were actually performed.

2 gm. of ergotinine were treated with 50 cc. of HNO_3 (1.4 sp. gr.). A vigorous reaction occurred. The mixture was then heated on the water bath for 18 hours. On dilution a definite odor of isobutyric acid was in evidence. The mixture was distilled over into dilute alkali but the amount of volatile material which was collected was too small in amount for study.

The orange-red acid solution which remained was evaporated on the bath to about 50 cc. On standing, clusters of pale yellow platelets separated. These were collected on a Jena funnel with nitric acid and then washed with water. The yield was 70 mg. After recrystallization from dilute acetone lustrous leaflets of *p*-nitrobenzoic acid separated, which melted at 235–236°, and showed no depression when mixed with *p*-nitrobenzoic acid.

3.815 mg. substance: 1.030 mg. H_2O, 7.052 mg. CO_2
3.870 "   "   : 0.292 cc. N (758.7 mm., 24°)
    $C_7H_5O_4N$. Calculated. C 50.28, H 3.02, N 8.39
         Found.   " 50.41, " 3.02, " 8.66

The mother liquor of the above crude *p*-nitrobenzoic acid was concentrated practically to dryness and left a considerable resinous

residue. This was treated with water and after standing additional crystalline material mixed with a brown-red resin deposited. After collection with water all that could be recovered from this material was an additional small quantity of impure p-nitrobenzoic acid.

The mother liquor from this second crude fraction was diluted somewhat and then extracted in a continuous extractor for 24 hours with ether. The aqueous layer which remained on concentration gave a small amount of the crystalline acid which softened above 280° after preliminary darkening. This substance was obtained in larger yield in later experiments as follows:

5 gm. of ergotinine were treated with a mixture of 120 cc. of HNO_3 (1.4) and 675 cc. of water and slowly distilled in an all-glass apparatus. The alkaloid rapidly became superficially colored a dark greenish brown and remained at first mostly as an undissolved resin. Shortly, a copious CO_2 formation could be readily detected with barium hydroxide. As the concentration of acid increased, the mixture became gradually a deep brown-red and small amounts of volatile material which crystallized in the receiver were carried over. The odor of this suggested somewhat p-nitrotoluene but the amount which could be collected was too small for identification. On reaching a stronger concentration of acid, the alkaloid had mostly dissolved and red fumes began to appear. At this point 400 cc. of water were again added and the distillation was resumed. On reaching the point of strong acid the dilution and concentration were repeated. These operations were repeated several times more. By this time the appearance of volatile solid material had practically ceased. Finally, the mixture was boiled down to the point of strong nitric acid when the odor of benzoic acid became evident in the distillate. At this point the operation was interrupted. The combined distillates were treated as given further on.

The deep brown-red acid solution was then concentrated *in vacuo* in an all-glass apparatus to remove the excess of nitric acid. The residue was dissolved in about 75 cc. of hot water. On standing, a small amount of red tar deposited. The supernatant liquor was decanted and concentrated to smaller volume. A crop of somewhat sulfur-yellow crystalline aggregates slowly deposited. After collection, this was recrystallized by solution in 100 cc. of boiling

water. On standing, faintly yellow aggregates of minute prisms slowly separated. The yield was 0.14 gm. The substance sintered above 280° after preliminary darkening. The mother liquor of this last recrystallization gave on concentration a second crop which was collected with water. It was found to be contaminated with considerable ether-soluble material and was therefore digested with ether and collected with this solvent. The yield was 50 mg. of this second crop which also softened above 280°. The ether washings on concentration gave 40 mg. of crystalline material which melted at 234° and proved to be p-nitrobenzoic acid.

The fact that the above high melting substance is practically insoluble in ether was then made use of in later experiments. 5 gm. of ergotinine were oxidized as previously. After final removal of the excess of nitric acid by concentration under diminished pressure the residue was dissolved in about 250 cc. of water. The brown-red mixture which contained suspended resin was placed in an extractor and continuously extracted with ether for 2 days, by which time no more color passed into the ether layer. The aqueous layer which was still brown-red and contained some suspended resin, was filtered and concentrated to small volume. Successive crops of crude crystalline acid were obtained, all of which softened at about 280–285°. The yield was 0.3 gm.

This was dissolved in 300 cc. of boiling water and cleared with norit. The filtrate was a faint straw-yellow. On concentration to about half and addition of dilute HCl the acid separated as almost colorless needles. It does not possess a characteristic melting point. On rapid heating it darkened above 265° and sintered together at about 290°, but did not melt even up to 350°. The substance is less soluble in dilute acid than in water. It is very sparingly soluble in the usual organic solvents. Its solution in dilute alkali, which is a pale yellow, exhibits a slight fluorescence. The substance gives only a faint pine splinter test.

14.322 mg. of substance were suspended in a few cc. of water and titrated against phenolphthalein with 0.1 N NaOH. Calculated for 3 equivalents, 1.346 cc.; found, 1.345 cc.

4.458 mg. substance: 1.085 mg. H_2O, 8.626 mg. CO_2
5.287 "   "   : 0.201 cc. N (759.2 mm., 25°)
4.760 "   "   : 3.275 mg. AgI
$C_{14}H_9O_8N$. Calculated. C 52.65, H 2.84, N 4.39, NCH_3 9.10
Found.   " 52.77, " 2.72, " 4.36,   " 8.51

The attempt to prepare a crystalline neutral methyl ester from the acid with diazomethane was unsuccessful. Only resinous material resulted. A monomethyl ester was prepared as follows: The acid was heated in a sealed tube for 18 hours with 50 parts of absolute methyl alcohol which contained 4 per cent of HCl. The crystalline powder was collected with methyl alcohol in which it is sparingly soluble. On heating, it behaves like the parent acid with no distinct melting point. It darkened above 260° and sintered at about 285° but did not melt below 350°.

4.050 mg. substance: 1.150 mg. H_2O, 7.970 mg. CO_2
3.700 "   "   : 2.555 "   AgI
$C_{15}H_{11}O_8N$.   Calculated.   C 54.04, H 3.33, OCH_3 9.31
              Found.   " 53.67, " 3.18,   "   9.13

The combined distillates obtained during the nitric acid oxidations were repeatedly extracted with ether. The latter was washed once with a little water and then extracted with dilute sodium hydroxide solution. The alkaline solution was acidified with sulfuric acid and as there was a pronounced odor of isobutyric acid the attempt was made to distil this into dilute alkali. The amount which distilled over, however, proved to be inadequate for definite identification. During this distillation a small amount of crystalline material was also carried over, which was obviously benzoic acid. This substance was also recovered from the acid solution which remained after the distillation of the volatile material. On cooling, a relatively very small amount crystallized. After recrystallization from ligroin it melted at 120–121°.

4.493 mg. substance: 1.998 mg. H_2O, 11.340 mg. CO_2
$C_7H_6O_2$.   Calculated.   C 68.82, H 4.96
              Found.   " 68.83, " 4.98

The ether solution which retained neutral material after the above extraction with alkali gave on concentration a small partly crystalline residue. The amount was too small for identification.

TOTAL NITROGEN OF THE BLOOD PLASMA OF NORMAL ALBINO RATS AT DIFFERENT AGES*

By PEARL P. SWANSON and ARTHUR H. SMITH

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Only certain of the organic constituents of the blood of the normal rat have been determined quantitatively. Previous data on the uric acid, non-protein nitrogen, and urea content have recently been augmented by Anderson, Honeywell, Santy, and Pedersen (1930). These authors repeated the earlier determinations for these components of the blood and added analyses for creatinine, creatine, and sugar. This investigation gives the most complete information available at the present time on the occurrence of organic constituents in the blood of the rat. In the present paper are reported data on the concentration of another constituent, the total nitrogen of the blood plasma at different ages. The only other study we have found bearing on this determination is that of Hatai (1918) on the refractive index of blood serum.

The data herein reported are based on the analyses of the blood of 119 normal albino rats. Each is the average of duplicate or triplicate determinations on the blood of *a single individual*. To obtain sufficient blood for the analysis, the animal was stunned slightly and an abdominal incision made. The visceral organs were then pushed aside and the blood was drawn from the abdominal aorta by means of a hypodermic syringe. No precautions were taken to prevent loss of CO_2. It is possible by this method to obtain as much as 6 cc. of blood from one adult rat. The time occupied by the entire procedure is short, frequently less than 90

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* Part of the data reported in this paper are taken from a dissertation presented by Pearl P. Swanson in partial fulfilment of the requirements for the degree of Doctor of Philosophy, Yale University, 1930.

seconds, and the great advantage of the method lies in the fact that the blood thus secured is entirely uncontaminated by other body fluids. Potassium oxalate was used as the anticoagulant. The possible error introduced in the use of oxalated plasma for analytical purposes is recognized, but every effort was made to treat all samples alike in the process of mixing with oxalate. (See Peters, Eisenman, and Bulger, 1925.) After centrifuging, the plasma was withdrawn by means of a tube drawn to a capillary with a U-shaped end. The aliquots for analysis were removed by means of a capillary pipette, calibrated to contain 0.45 cc. On the basis of data presented by Peters and Van Slyke (1931), a sample of plasma of this size contains approximately only 5 mg. of nitrogen; it was necessary, therefore, to select a method of analysis that would yield reliable results with this small quantity of material. If all manipulative procedures were carefully standardized, the macro-Kjeldahl-Gunning method (Association of Official Agricultural Chemists, 1919) was found satisfactory. For example, it was necessary to consider the following items in the standardization of the technique: the method of measuring the sample, the delivery of the same into the Kjeldahl flask, the delivery of the sulfuric acid, the time of digestion, the dilution of the digest, the quantity of zinc employed, the quantity of indicator in the receiving flask, the size of the aliquot of standard acid used, and the establishment of the end-point in the final titration. The necessity for this standardization of procedure is clearly illustrated by two series of determinations for nitrogen on aliquots drawn from two large samples of pooled plasma designated as Plasma 1 and Plasma 2, respectively. Before the manipulative steps were all definitely controlled, twenty-one analyses were made on aliquots from Plasma 1. The average concentration of nitrogen in 0.45 cc. of this plasma was 5.28 mg. with a probable error of 0.057 mg. which is 1.08 per cent of the mean. The probable error was estimated by Fisher's (1930) scheme for the treatment of small samples. The range of the determinations was 4.71 to 6.02 mg. Under these conditions, the method was not highly reliable.

On the other hand, when the procedure was finally standardized in all respects, the average of twelve aliquot determinations on Plasma 2 was 5.46 mg. with a probable error of 0.018 mg. and a

percentage probable error of 0.33. The range of the determinations in this instance, was only 5.36 to 5.59 mg. of nitrogen. Improvement in the accuracy of the method is indicated by the reduction in the percentage probable error from 1.08 to 0.33 per cent.

The method was next checked by analyzing for the nitrogen present in aliquots of an aqueous solution of resublimed NH_4Cl calculated to contain 1.32 mg. of nitrogen per cc. The recovery of nitrogen from two 1 cc. samples of the reagent was 1.32 mg. and 1.34 mg. respectively.

The procedure as finally adopted may be described as follows:

The plasma was measured into 800 cc. flasks with the calibrated capillary pipette, the tip of which was carefully wiped before the final adjustment of volume was made. After delivery, it was rinsed by repeated washings with a measured volume of water. The plasma was digested with 20 cc. of concentrated H_2SO_4, 2 cc. of 5 per cent $CuSO_4$ solution, and 10 gm. of K_2SO_4. Since the acid is the reagent carrying the most contaminating nitrogen, extreme care was taken in its measurement. Digestion was continued for 1 hour after the solution cleared. The digest was always diluted with the same quantity of water. A glass spoon, calibrated to hold only 6 to 10 granules, was used to measure the granulated zinc added to prevent bumping. A quantity of zinc in excess of this amount produced an evolution of hydrogen that caused the formation of a spray which introduced error. The distillate was collected in 35 cc. of 0.0357 N HCl diluted with 50 cc. of water. This allowed approximately 20 cc. of titratable acid, thus reducing error at this point. 3 drops of methyl red were added. Distillation was continued until the receiving flask contained exactly 250 cc. of liquid. When titrating, the point at which the solution became colorless was chosen for the end-point. A fraction of a drop of either the 0.0357 N acid or alkali would turn the solution distinctly pink or yellow, respectively. Corrections were made for the nitrogen present in the reagents by running duplicate blank determinations for every set of samples analyzed.

The rats used for the study were reared upon adequate diets, either the stock ration for the colony or a synthetic diet known to be adequate according to present day standards. For the most part, the animals used were males. A few of the rats in the

Total Nitrogen Content of Plasma of Rats at Different Ages

The results are expressed as gm. per 100 cc. of plasma.

No. of analyses	21–28	37	50	60	80	100	130	180	360
					Age, days				
1	0.83	0.98	1.07	1.09	0.97	1.14	1.22	1.20	1.16
2	0.77	0.84	1.01	1.03	1.08	1.04	1.07	1.19	1.29
3	0.88	0.99	1.06	1.06	1.16	1.00	1.09	1.33	1.25
4	0.94	0.87	0.96	1.08	1.11	1.08	1.15	1.21	1.43
5	0.89	0.90	0.88	0.94	1.03	0.89	1.14	1.20	1.20
6	0.83	0.82	1.01	1.01	1.17	1.03	1.19	1.07	1.06
7	0.84	0.95	0.93		0.98	1.08	1.19	1.13	1.29
8	0.75	0.92	1.07		0.99	0.98	1.14	1.12	1.25
9	0.67	0.87	1.03			0.98	1.13		1.23
10	0.70		1.13			1.10	1.06		1.22
11	0.77		1.05				1.11		1.17
12	0.76		0.96				1.04		
13	0.75		1.01				1.29		
14	0.78		1.01				1.09		
15	0.76		0.94				1.14		
16	0.86		1.03				1.12		
17	0.78		1.06				1.29		
18	0.84		1.08				1.28		
19	0.85		1.09				1.10		
20	0.83		0.93				1.07		
21	0.85		0.95				1.10		
22	0.85								
23	0.83								
24	0.83								
25	0.70								
Mean	0.81	0.90	1.01	1.04	1.06	1.03	1.14	1.18	1.23
Standard deviation	±0.06	±0.06	±0.06	±0.05	±0.08	±0.10	±0.07	±0.07	±0.09
Probable error of mean	±0.008	±0.013	±0.009	±0.015	±0.019	±0.015	±0.010	±0.019	±0.019

21 to 28 days and in the 360 days groups were females. The number of animals studied in each age group varied from six to twenty-five. No attempt was made to restrict the food consumed before the withdrawal of blood. The results are recorded in Table I.
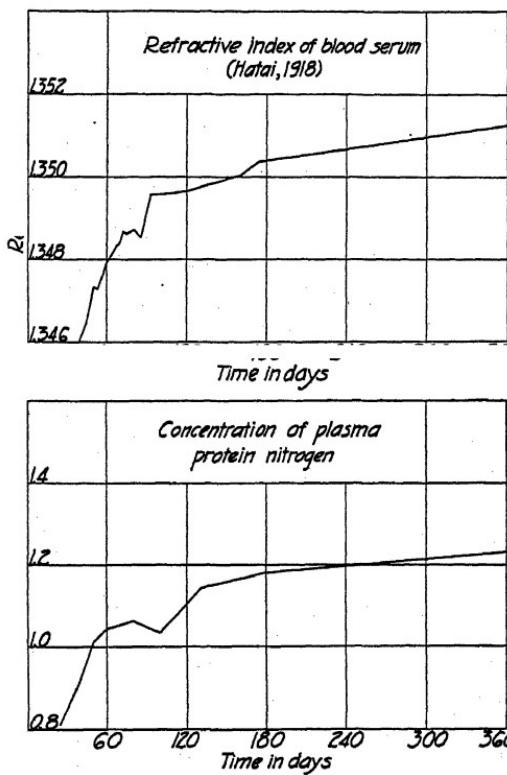
FIG. 1. Showing the effect of age on the refractive index of the blood serum of the albino rat and on the concentration of the plasma protein nitrogen.

An inspection of Table I shows that rat plasma is similar to human plasma as regards total nitrogen content (Peters and Van Slyke, 1931). There is an increase in the total nitrogen concentration of the plasma from 0.81 gm. per 100 cc. of plasma at weaning (21 to 28 days) to 1.22 gm. when the rats are 360 days old. The most pronounced augmentation occurs in early life; *i.e.*, from wean-

ing until the rats are 50 days old. Thereafter, the increments in plasma nitrogen concentration are very gradual and the data indicate that an approximately uniform level is maintained in adult life.

There is only one definite break in the gradual increase of the concentration of nitrogen in the plasma (Fig. 1). The decrement occurs at 100 days but is predicted in the determinations made when the rats were 60 and 80 days old respectively. Hatai (1918) has similarly shown (see Fig. 1) that the rise in the refractive index of the serum becomes irregular at approximately this same interval in the life history of the rat. He believes that this period, representing as it does the onset of sexual maturity, is one in which important changes occur in the composition of the serum. In this event, it would seem that our rats, reared largely on synthetic diets, developed more slowly than did the rats studied by Hatai. In both instances, subsequent alterations are consistent and gradual. Although the differences in the average concentration of the nitrogen in the plasma at the age intervals studied in the present investigation are not significant in all cases, the trend in the change of the concentration of the constituent is highly so; probably a natural phenomenon associated with advancing age (Moulton, 1923) and related to the increasing content of total solids found in the blood of the rat as it becomes older (Hatai, 1918).

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ON THE STRUCTURE OF THE SO CALLED 5-METHYL GLUCOSE OF OHLE AND VON VARGHA

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In continuation of the work on the nature of the glycoside formation in the partially substituted sugars,[1] a study of the 5-methyl glucose described by Ohle and von Vargha[2] was undertaken by us. On the basis of previous experience, a substance of this structure was expected to form but one glucoside, the furanoside, and it was therefore surprising to find indication of the formation of two glucosides. These were formed, moreover, at approximately the same rates as in the case of unsubstituted glucose. The formation of two glucosides from 5-methyl glucose would require either the existence of glucosides with ring structures other than $< 1, 4>$ and $< 1, 5>$, or else the migration of the methyl group in the course of glucoside formation. Further study, however, has convinced us that no irregularity had occurred but that instead an incorrect structure has been assigned by Ohle to his substance, which in reality is 6-methyl glucose.

The following observations led to this conclusion. The osazone of the substance described by Ohle has a melting point (180–185°) which is very close to that last reported for 6-methyl glucosazone (184–187° corrected).[3] The rotation of the osazone of the "5-methyl" glucose was reported as $-101.9°$ in pyridine,[2] and this we have confirmed. However, in alcohol the "5-methyl" glucosazone has initial and final rotations of $-70°$ and $-44°$ respectively. These values are practically identical with those reported by

[1] Levene, P. A., Raymond, A. L., and Dillon, R. T., *J. Biol. Chem.*, **96**, 449 (1932).

[2] Ohle, H., and von Vargha, L., *Ber. chem. Ges.*, **62**, 2435 (1929).

[3] Helferich, B., and Günther, E., *Ber. chem. Ges.*, **64**, 1276 (1931).

Helferich and Günther[3] and by Ohle and von Vargha[4] for 6-methyl glucosazone; namely, initial $-70.3°$ and $-69.6°$, and final, $-46.9°$ and $-46.4°$.

Methylation of the methylglucoside resulting after long heating of a solution of Ohle's "5-methyl" glucose in methyl alcohol containing hydrogen chloride, gave a tetramethyl methylglucoside which was hydrolyzed to a tetramethyl glucose. This was crystallized and found to have the properties of 2,3,4,6-tetramethyl glucose. The tetramethyl glucose was further identified by conversion to the anilide, identical with that from 2,3,4,6-tetramethyl glucose. From 5-methyl glucose this substance could have been formed only by actual migration of the methyl group from position (5), an improbable assumption. Moreover, this possibility is contradicted by the curves of lactone formation of the "5-methyl" gluconic acid which indicate two lactones, formed at rates which are close to those found for the corresponding lactones of unsubstituted glucose. As the lactone formation was measured at room temperature and in only slightly acid solution, migration of a methyl group would appear most unlikely.

For further evidence, the monomethyl glucose was converted to the tetracetate, and through the acetobromo derivative, to the triacetyl methyl methylglucoside. Both of these derivatives were found to have the properties reported by Helferich and Günther for the corresponding derivatives of 6-methyl glucose.

All of these results lead to the conclusion that the crystalline "5-methyl" glucose described by Ohle and von Vargha is the 6-methyl glucose, previously obtained by Helferich and Becker,[5] though in amorphous state. As a consequence of this conclusion, the structure of the "trimethyl glucose" of Pacsu,[6] shown by Shinle[7] to be a monomethyl glucose, was in need of reinvestigation inasmuch as the 5-methyl structure was excluded by Schinle on the basis of the claims of Ohle. However, it will be shown in a subsequent publication that the assumption of Shinle is probably correct.

Work on the synthesis of 5-methyl glucose is now in progress.

[4] Ohle, H., and von Vargha, L., *Ber. chem. Ges.*, **62**, 2425 (1929).

[5] Helferich, B., and Becker, J., *Ann. Chem.*, **440**, 1 (1924).

[6] Pacsu, E., *Ber. chem. Ges.*, **57**, 849 (1924); **58**, 1455 (1925).

[7] Schinle, R., *Ber. chem. Ges.*, **65**, 315 (1932).

Methyl Monoacetone Glucose—6-Tolylsulfo monoacetone glucose was prepared from monoacetone glucose following the procedure of Ohle and von Vargha.[2]   In general the yields were not quite as high as those reported by them.

The 6-tolylsulfo monoacetone glucose was converted to the methyl monoacetone glucose by a slight modification of Ohle's[2] procedure, the isolation of the anhydromonoacetone glucose being omitted.   50 gm. of tolylsulfo monoacetone glucose were dissolved in 100 cc. of methyl alcohol, cooled in an ice-salt mixture, and added with vigorous shaking to a solution of 3.75 gm. of metallic sodium in 100 cc. of methyl alcohol similarly cooled in an ice-salt mixture.   The product was allowed to stand 20 minutes in the freezing mixture and an ice-cold solution of 7.5 gm. of metallic sodium in 125 cc. of methyl alcohol was then added with shaking. A separation of sodium toluenesulfonate occurred immediately. The mixture was allowed to stand at room temperature with occasional shaking for 2 days and was then cooled in ice water. Hydrochloric acid was added until the solution became just acid to phenolphthalein (pH about 7.6).   2 volumes of ether were added and the precipitate was filtered off and washed with more ether. The filtrate and washings were combined and concentrated under reduced pressure to a syrup which was taken up in chloroform. The chloroform solution was washed twice with water, dried with sodium sulfate, and concentrated under reduced pressure to a syrup.   This was taken up in ether and the solution was cooled in the refrigerator.   Pentane was added just to turbidity and the mixture was seeded.   The seed crystals were obtained by distilling a portion of the material under greatly diminished pressure as described by Ohle.   The yield of crude crystalline product was 19 gm. and after one recrystallization from ether-pentane, 15 gm.

Several lots of the methyl monoacetone glucose were combined and recrystallized as above.

The substance had the composition of a methyl monoacetone hexose.

4.595 mg. substance: 8.635 mg. CO_2 and 3.225 mg. H_2O
3.395   "   "   : 3.475 "   AgI
$\quad$ $C_{10}H_{18}O_6$.   Calculated.   C 51.26, H 7.76, OCH_3 13.24
$\quad$ 234.1   Found.   " 51.24, " 7.85, "   13.51

The product sintered at about 70° and then melted at 71–72°.
It had a rotation, in chloroform, of $[\alpha]_D^{20} = \dfrac{-0.24° \times 100}{2 \times 2.0} = -6.0°$.
Ohle and von Vargha[2] give 71–72° as the melting point and −6.4°
as the rotation.

Monomethyl Glucose—The methyl monoacetone glucose de-
scribed above was hydrolyzed exactly as described by Ohle and
von Vargha[2] and the product was recrystallized twice from
absolute alcohol. The composition corresponded to a mono-
methyl hexose.

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4.725 mg. substance: 7.511 mg. CO₂ and 3.040 mg. H₂O
5.891  "       "      : 7.435  "  AgI
        C₇H₁₄O₆.  Calculated.  C 43.28, H 7.27, OCH₃ 15.93
        194.1     Found.       " 43.34, " 7.19, "  16.66
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The substance had a melting point which varied greatly with the
rate of heating. If kept at 138° for several minutes it melted to a
clear liquid, but if heated rapidly, it softened at 150° and melted
at 153–154°. Ohle and von Vargha[2] reported 143–144° as the
melting point. The optical rotation in water of our product was
101.8° 1 minute after dissolving, 86.0° at 30 minutes, 59.5° at 3
hours, and 56.0° at 5 and at 24 hours. A second sample had an
initial rotation of 104.5° and an equilibrium rotation, after 5
hours, of 58.5°. Ohle and von Vargha[2] reported 101.2° 3 minutes
after dissolving, and 59.92° at equilibrium after 3 hours.

Methyl Glucosazone—0.5 gm. of the methyl glucose was converted
to the osazone exactly as described by Ohle and von Vargha.[2]
The product, which formed readily, was recrystallized from
alcohol, in which it was fairly soluble.
The analysis corresponded to a methyl hexosazone.

```
4.880 mg. substance: 0.651 cc. N (28° and 761 mm.)
4.650  "      "      : 3.070 mg. AgI
        C₁₉H₂₄O₄N₄.  Calculated.  N 15.06, OCH₃ 8.33
        372.2        Found.       " 15.14, "  8.72
```

On heating the recrystallized material, it contracted at 180°,
melted at 183°, and decomposed at 192°. Ohle and von Vargha[2]
had reported 180° with decomposition as the melting point of their
material. Helferich and Günther[3] gave 184–187° (corrected) as
the melting point of the pure 6-methyl glucosazone, and Ohle and

von Vargha[4] found 178° as the melting point of the osazone of 6-methyl glucose prepared through isodiacetone glucose.

The rotation of the product, in pyridine, was −102° initial, and −28° at equilibrium after 24 hours. Ohle and von Vargha[2] reported −101.9° initial, but were unable to observe the end value due to coloration of the solution. Our own solution darkened only slightly in 6 days. The rotation of our product in absolute alcohol was −70° 5 minutes after dissolving and −44° at equilibrium after 24 hours. Helferich and Günther[3] reported −70.3° initial and −46.9° at equilibrium for the rotations, in alcohol, of the osazone of 6-methyl glucose, and Ohle and von Vargha[4] reported −69.6° initial and −46.4° at equilibrium for the osazone of 6-methyl glucose prepared from methyl isodiacetone glucose.

Tetracetyl-6-Methyl Glucose—3 gm. of the "5-methyl" glucose were acetylated according to the procedure described by Helferich for the 6-methyl glucose. The product crystallized easily and was twice recrystallized from a little absolute alcohol. The composition corresponded to a tetracetyl methyl hexose.

4.695 mg. substance: 8.568 mg. CO_2 and 2.550 mg. H_2O
6.075 "   "   : 4.001 " AgI
$C_{15}H_{22}O_{10}$.   Calculated.   C 49.70, H 6.12, OCH_3 8.55
362.2   Found.   " 49.76, " 6.07,   "   8.69

The melting point was 95–96° and the rotation, in chloroform, was $[\alpha]_D^{20} = \dfrac{+0.86° \times 100}{2 \times 2.0} = +21.5°$. Helferich reports 91–93° (corrected) as the melting point and +20.9° as the rotation, in chloroform, for β-tetracetyl-6-methyl glucose.

Triacetyl-6-Methyl-β-Methylglucoside—The syrupy tetracetate from 9.5 gm. of the "5-methyl" glucose was treated with 50 cc. of a saturated solution of hydrogen bromide in glacial acetic acid without preliminary attempts at crystallization. After standing for 2 hours at room temperature the mixture was poured onto ice, ice water was added, and the solution was extracted three times with chloroform. The extracts were washed three times with ice water, dried with sodium sulfate, and concentrated under reduced pressure to a thick syrup. This was taken up in 100 cc. of dry methyl alcohol, cooled to 10°, and 10 gm. of dry silver oxide were added. The mixture was shaken vigorously. A test after 10

minutes indicated that practically no bromide remained. The mixture was filtered, then refiltered with charcoal, and concentrated under reduced pressure to a syrup. This was dissolved in ether and cooled. Pentane was added just to turbidity. On standing overnight in the ice box the material crystallized. A second crop was obtained on concentrating the mother liquors. The total yield was 12.4 gm. The material was recrystallized twice from absolute alcohol and once from ether.

The composition corresponded to a triacetyl methyl methylhexoside.

5.001 mg. substance: 9.195 mg. CO_2 and 2.920 mg. H_2O
4.775 "   "   : 6.685 " AgI
        $C_{14}H_{22}O_9$. Calculated. C 50.27, H 6.64, OCH_3 18.55
        334.2   Found.   " 50.17, " 6.53, "   18.48

The product melted at 104–105° and had a rotation, in chloroform, of $[\alpha]_D^{20} = \dfrac{-0.58° \times 100}{2 \times 2.0} = -14.5°$. Helferich and Günther[3] give a melting point of 107–108° and a rotation of $-12.4°$ for the triacetyl-β-methylglucoside of the 6-methyl glucose.

Methyl Methylglucoside—5 gm. of the monomethyl glucose were dissolved in 250 cc. of dry methyl alcohol containing 0.5 per cent hydrogen chloride. The solution was sealed in a round bottom flask and heated in a bath of boiling carbon tetrachloride for 43 hours. The specific rotation of the solution, after cooling, was +115°. The hydrogen chloride was removed with silver oxide and the silver precipitate was washed with warm methyl alcohol. The methyl alcohol solution and washings were combined and concentrated under reduced pressure to a syrup. Of this syrup 0.4 gm. were removed for analysis and dried over phosphorus pentoxide under greatly reduced pressure. Although not a pure product, the composition corresponded fairly well to a methyl methylhexoside.

3.452 mg. substance: 5.700 mg. CO_2 and 2.500 mg. H_2O
3.710 "   "   : 9.170 " AgI
        $C_8H_{16}O_6$. Calculated. C 46.15, H 7.76, OCH_3 29.81
        208.1   Found.   " 45.02, " 8.10, "   32.62

Tetramethyl Methylglucoside—The product prepared above was methylated by the Purdie method with 45 cc. of methyl iodide as the solvent. The solution was initially turbid. The product was

isolated in the usual manner by repeated extraction with hot chloroform. The solution was concentrated under reduced pressure to a syrup and this was redissolved in 45 cc. of methyl iodide, giving a clear solution. After remethylation the product was isolated in the same manner. The chloroform extract was concentrated under reduced pressure to a syrup and this was distilled under greatly reduced pressure. Yield 4.2 gm. The product had a rotation, in alcohol, of $[\alpha]_D^{20} = +101°$, and a refractive index of $n_D^{19} = 1.4413$. The composition corresponded to a tetramethyl methylhexoside.

5.980 mg. substance: 11.455 mg. CO_2 and 4.685 mg. H_2O
4.670   "   "   : 21.311 "   AgI
   $C_{11}H_{22}O_6$. Calculated. C 52.76, H 8.86, OCH_3 61.95
   250.2   Found.   " 52.24, " 8.76,   "   60.23

Tetramethyl Glucose—3.8 gm. of the above product were dissolved in 75 cc. of 1.5 N hydrochloric acid and heated on the steam bath for 7 hours. The hydrochloric acid was removed with silver oxide and the silver salts were extracted once with warm water and twice with boiling acetone. The combined extracts were concentrated under reduced pressure to a syrup. On seeding with a crystal of 2,3,4,6-tetramethyl glucose, the syrup crystallized immediately. The product was twice recrystallized from pentane containing 0.5 per cent of ether. It melted at 88–90° and had a specific rotation, in alcohol, of 102° 2 minutes after dissolving, and 83.5° at equilibrium after 48 hours. The melting points recorded in the literature range from 88–104°. The rotation in alcohol is reported as 104.9° initial, and 83.9° at equilibrium.

Tetramethyl Glucose Anilide—0.5 gm. of the above tetramethyl glucose was dissolved in 2 cc. of absolute alcohol, 0.58 cc. of freshly distilled aniline (3 mols) were added, and the mixture was refluxed on the steam bath for 3 hours. On cooling, the product crystallized. It was filtered off, washed with a little absolute alcohol, and recrystallized from absolute alcohol. It melted at 137–138° and had a rotation, in methyl alcohol containing 0.001 per cent hydrochloric acid, of +64.0° at equilibrium. Greene and Lewis[8] report a melting point of 138° and a final rotation of +59° in methyl alcohol containing 0.001 per cent hydrochloric acid.

[8] Greene, R. D., with Lewis, W. L., *J. Am. Chem. Soc.*, **50**, 2813 (1928).

Methyl Gluconic Acid and Lactone Formation—To 5 gm. of the methyl glucose in 90 cc. of water, was added a solution of 12.5 gm. of iodine and 25 gm. of barium iodide in 60 cc. of water. The mixture was stirred mechanically and 300 cc. of 0.5 N barium hydroxide solution were dropped in, 20 minutes being required for the addition. The mixture was stirred for an additional 15 minutes and then acidified with sulfuric acid. Sulfur dioxide was passed in until the iodine was exactly reduced, and the halide was removed by adding moist silver carbonate. Acid was added as required to keep the mixture acid to Congo paper. The silver precipitate was filtered off, resuspended in dilute sulfuric acid, refiltered, and washed with dilute sulfuric acid. The combined filtrates were saturated with hydrogen sulfide, and aerated to remove

TABLE I

Observed Rotations during Lactone Formation

Time	α	Time	α	Time	α
min.	*degrees*	*hrs.*	*degrees*	*hrs.*	*degrees*
$1\frac{1}{2}$	0.00	1	0.54	72	1.10
3	0.09	$1\frac{1}{2}$	0.53	144	1.27
8	0.18	3	0.57	288	1.24
15	0.35	6	0.61		
32	0.45	23	0.85		

the excess. A warm saturated solution of barium hydroxide was added to a pH of 8.5 and the mixture of silver sulfide and barium sulfate was filtered off and washed with water. The combined filtrate and wash water were concentrated under reduced pressure to about 100 cc., readjusted to pH 8.5, and filtered with charcoal. This filtrate was concentrated to a thick syrup and then dried over phosphorus pentoxide to a glassy solid. This was powdered and redried to constant weight. Yield 6.7 gm. The composition corresponded to a barium salt of a methyl hexonic acid.

8.530 mg. substance: 7.315 mg. AgI
12.090 "   "   : 5.490 " $BaSO_4$
        $(C_7H_{13}O_7)_2Ba$. Calculated. OCH_3 11.16, Ba 24.74
        555.6   Found.   "   11.31, " 26.72

The rotation, in water, was $[\alpha]_D^{21} = \dfrac{+1.06° \times 100}{2 \times 2.0} = +26.5°$.

0.250 gm. of this product was dissolved in water, 0.97 cc. (2 mols per mol of Ba) of 1.0 N hydrochloric acid was added, the mixture was diluted immediately to a volume of 5.0 cc., and the rotation
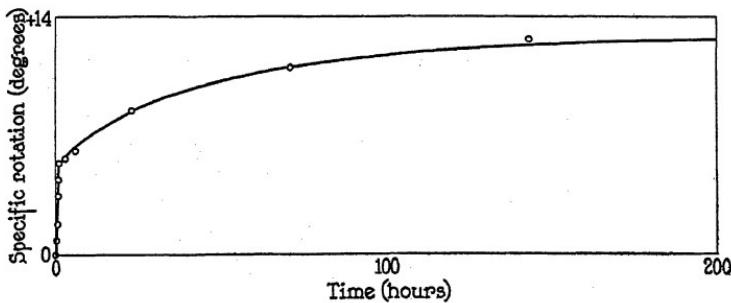
FIG. 1. Optical rotation during lactone formation at 25°
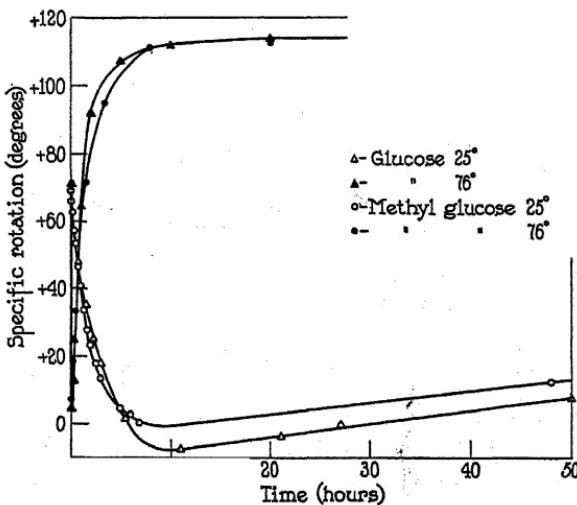
FIG. 2. Optical rotation during glucoside formation at 25° and at 76°. Glucose and methyl glucose.

was measured in a 2 dm. tube with sodium (D) light. The observed rotations are given in Table I and the specific rotations (calculated on the basis of barium salt) are plotted in Fig. 1.

Glucoside Formation—The methods and technique have been

previously described[9] and need not be repeated in detail here. A 0.344 molal solution of the sugar in dry methyl alcohol containing 0.5 per cent of dry hydrogen chloride was used. Experiments were made at room temperature, and in sealed tubes heated in a bath of boiling carbon tetrachloride. The rotations were measured at 20° with sodium (D) light. 4 dm. tubes were used for the room temperature experiments and 2 dm. for the others. The specific rotations calculated from the data are plotted in in Fig. 2. The corresponding curves for glucose are reproduced from an earlier paper for purposes of comparison.

TABLE II

Glucoside Formation at 25° from Reduction Determinations

Time	Cc. 0.01 N thiosulfate		Mg.		Free sugar, per cent		Distribution of sugar, per cent		
	Before hydrolysis	After hydrolysis	Before hydrolysis	After hydrolysis	Before hydrolysis	After hydrolysis	Free	Glucoside	
								γ	Normal
hrs.									
0	10.88	7.03	10.55	6.82*	106	102	106	−4	−2
1	7.86	6.93	7.62	6.73*	76	101	76	25	−1
3	4.32	6.90	4.19	6.70*	42	100	42	58	0
7	2.80	6.74	2.72	6.54*	27	98	27	72	2
24	2.39	8.82	2.32	8.55	23	86	23	63	14
48	2.47	7.64	2.40	7.41	24	74	24	50	26

* The total sugar present in these samples was 6.67 mg. In all the others it was 10.0 mg.

In order to determine analytically the character of the glucosides which were formed, the method previously developed[1, 9] was used. This consisted in determining the amount of reducing sugar present in a particular sample, before and after hydrolysis with dilute acid. The difference is assumed to represent the amount of γ-glucoside present in the sample. The limitations of the method, as well as the necessary corrections, have been previously discussed in detail.

The micro Willstätter[1] method was used without modification. This was found to give practically theoretical values for the

9 Levene, P. A., Raymond, A. L., and Dillon, R. T., *J. Biol. Chem.*, **95**, 699 (1932).

methyl glucose both before and after heating with acid, so that no correction was required. A sample of the glucoside which had been formed by heating the acid methyl alcohol solution at 76° for 8 hours was hydrolyzed with 0.1 N hydrochloric acid at 100° for 10 minutes and then analyzed for reducing sugar. The amount present was, within the limits of experimental error, no more than before the hydrolysis so that this correction was likewise omitted.

The data, and the amounts of γ-glucoside, normal glucoside, and free sugar, calculated from them are given in Table II. Comparison with the results on glucose in the earlier papers[1,9] shows that the rates of formation of the γ-glucosides are closely alike in the two sugars, as are those for the normal glucosides.

THE SUBSTITUTION OF GLUCOSE IN POSITION (4). I

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For some time we have been desirous of having intermediates for the synthesis of glucose derivatives substituted in position (4), in particular for the synthesis of 4-methyl glucose, 4-phospho glucose, and of certain disaccharides. Schinle[1] has recently ascribed the structure of 4-methyl glucose to the "trimethyl" glucose of Pacsu,[2] but the structure has not been well established, and the method of preparation is such as to be of little value for the synthesis of derivatives other than the methyl glucose. Helferich[3,4,5] has also described two possible intermediates, 1,2,3,6-tetracetyl glucose and 2,3,6-triacetyl-β-methylglucoside, but their preparation (by isomerization of the corresponding 2,3,4 derivatives) is too complicated.

The procedures we have been engaged in developing are similar in certain respects to those published by Oldham and Rutherford[6] but avoid the tedious introduction and removal of the tolylsulfo groups. Benzylidene-β-methylglucoside has been shown to be 4,6-benzylidene,[7] and as the ring is in position (5), positions (2) and (3) are unoccupied. By benzoylation of this derivative and subsequent removal of the benzylidene group by careful hydrolysis,

[1] Schinle, R., *Ber. chem. Ges.*, **65**, 315 (1932).

[2] Pacsu, E., *Ber. chem. Ges.*, **57**, 849 (1924); **58**, 1455 (1925).

[3] Helferich, B., and Klein, W., *Ann. Chem.*, **450**, 219 (1926); **455**, 173 (1927).

[4] Helferich, B., Bredereck, H., and Schneidmüller, A., *Ann. Chem.*, **458**, 111 (1927).

[5] Helferich, B., and Bredereck, H., *Ber. chem. Ges.*, **64**, 2411 (1931).

[6] Oldham, J. W. H., and Rutherford, J. K., *J. Am. Chem. Soc.*, **54**, 366 (1932).

[7] Ohle, H., and Spencker, K., *Ber. chem. Ges.*, **61**, 2387 (1928).

2,3-dibenzoyl-β-methylglucoside was obtained. This substance, on partial benzoylation, gave, in good yield, a crystalline tribenzoate which appears to be 2,3,6-tribenzoyl-β-methylglucoside. This conclusion rests largely upon the fact that the acetyl derivative of this tribenzoate is not identical with the known 6-acetyl-2,3,4-tribenzoyl-β-methylglucoside already known.

The tribenzoate, upon methylation, yielded a product which has not yet been brought to crystallization. Debenzoylation with barium oxide in methyl alcohol, and reacetylation, gave a crystalline product with the composition of a methyl triacetyl methylglucoside. If the benzoate is really 2,3,6-tribenzoyl-β-methylglucoside, then this product should be triacetyl-4-methyl-β-methylglucoside. Schinle, as mentioned above, considers Pacsu's "trimethyl" glucose to be 4-methyl glucose and we have therefore prepared some of this substance and have converted it, through the acetobromo compound, into the methyl triacetyl-β-methylglucoside. The product is identical with the derivative described above.

Inasmuch as in the case of Schinle's substance the choice of the structure lies between that of 4-methyl and 5-methyl glucose,[8] and as our substance, being derived from a normal glucoside, cannot be substituted in position (5), it seems justifiable to conclude that both products, the one by Schinle and the one by us, have the structure of 4-methyl-β-methylglucoside.

Although the tribenzoate described above is useful for methylation, the relative stability of the benzoyl groups renders the substance unsuitable for the preparation of certain derivatives, such as the phosphoric ester. For this purpose advantage may be taken of the corresponding acetyl compounds. 2,3-Diacetyl-β methylglucoside was prepared by careful hydrolysis of the 2,3-diacetyl-4,6-benzylidene-β-methylglucoside, and was then partially acetylated, giving in good yield a crystalline triacetyl-β-methylglucoside. This was found to have the same melting point

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[8] Schinle assigned this structure since the methyl glucose was not identical with the already described 2-, 3-, 5-, and 6-methyl glucoses. We have since shown that the so called 5-methyl glucose of Ohle and von Vargha is really 6-methyl glucose (Levene, P. A., and Raymond, A. L., *J. Biol. Chem.*, **97**, 751 (1932)) so that the 5-methyl structure may again be regarded as a possibility for the monomethyl glucose from the mercapto derivative.

as the 2,3,6-triacetyl-β-methylglucoside of Helferich, mentioned above, but the rotation was somewhat different ($-59.0°$ as against the value $-64.9°$ reported by Helferich[4]). The p-tolylsulfo derivative of our triacetyl compound had a rotation and melting point which agreed quite well with those reported by Helferich for the corresponding p-tolylsulfo derivative of his triacetyl compound, and not at all with those for the triacetyl-6-p-tolylsulfo-β-methylglucoside. It thus seems probable that our triacetyl derivative is identical with that of Helferich.

We may mention that in addition to the acetyl derivative of the tribenzoate, the p-tolylsulfo tribenzoyl-β-methylglucoside was prepared, but attempts at debenzoylation led to the extensive removal of the tolylsulfo group so that this substance was not further utilized.

The dibenzoyl glucoside, prepared as an intermediate, has not yet been crystallized. Josephson,[9] on the other hand, prepared a substance to which he tentatively ascribed the structure of 2,3-dibenzoyl-β-methylglucoside, and which he obtained in crystalline form. He also described the diacetate, 2,3-dibenzoyl-4,6-diacetyl-β-methylglucoside, melting point 166°. The diacetate of our syrupy dibenzoyl compound, obtained crystalline and in excellent yield, melts at 131–132°, so that we are inclined to question the structures assigned by Josephson to his compounds.

We thus appear to have convenient methods for the preparation of 2,3,6-triacetyl and tribenzoyl-β-methylglucosides. We intend, first, to obtain further confirmation of their structure, and secondly, to make use of them for the synthesis of 4-phospho glucose, of 4-methyl glucose, and of disaccharides.

<div align="center">EXPERIMENTAL</div>

2,3-Dibenzoyl-β-Methylglucoside—β-Methylglucoside was converted to the benzylidene derivative, and benzoylated as described in the literature.[7] The 2,3-dibenzoyl-β-methylglucoside was recrystallized and then hydrolyzed. 10 gm. were dissolved in 90 cc. of acetone and 10 cc. of 1 N hydrochloric acid were added. The mixture was refluxed on the steam bath for 5 hours (the optical rotation was constant after 4 hours) and then cooled. The acid

[9] Josephson, K., *Ber. chem. Ges.*, **62**, 317 (1929).

was removed by shaking with silver oxide, and the filtrate from this was refiltered with charcoal. After concentrating under reduced pressure until two layers formed, the mixture was three times extracted with pentane, which was decanted off. The remaining product was taken up in chloroform and washed with sodium bisulfite solution to remove the last traces of benzaldehyde. After washing twice with water, the chloroform solution was dried with sodium sulfate and concentrated to a syrup. A portion of this was dried over phosphorus pentoxide under greatly reduced pressure, powdered, and redried. Although not a crystalline product, the analysis corresponded fairly well to a dibenzoyl methylhexoside.

4.856 mg. substance: 11.075 mg. CO_2 and 2.390 mg. H_2O
5.500 "   "   : 3.530 " AgI
$C_{21}H_{22}O_8$.   Calculated. C 62.66, H 5.52, OCH_3 7.71
402.2   Found.   " 62.20, " 5.50, "   8.47

As yet this substance has not been crystallized. Its rotation, in chloroform, was $[\alpha]_D^{20} = \dfrac{+3.71° \times 100}{2 \times 2.0} = +92.8°$. Josephson[9] did not report the rotation of his supposed 2,3-dibenzoyl-β-methylglucoside.

2,3-Dibenzoyl-4,6-Diacetyl-β-Methylglucoside—The syrupy dibenzoate from the hydrolysis of approximately 5.5 gm. of dibenzoyl-benzylidene-β-methylglucoside, was dissolved in 50 cc. of dry pyridine, and 4 cc. of acetic anhydride were added. The mixture warmed somewhat, and without cooling, was left for 18 hours at room temperature. 5 cc. of water were added, and after standing for $\frac{1}{2}$ hour, ice water was added and the mixture was extracted three times with chloroform. The combined extracts were washed once with water, then with dilute, ice-cold sulfuric acid until acid to Congo paper, and then twice more with water. After drying with sodium sulfate, the chloroform extract was concentrated under reduced pressure to a thick syrup. The syrup was dissolved in a little warm ether, and on cooling this extract the product crystallized immediately. Pentane was added and the mixture was allowed to stand in the ice box. The crystals were filtered off and the solution was concentrated and treated in the same manner to secure a second crop. The combined product

weighed 5.1 gm. It was three times recrystallized from methyl alcohol. Its composition corresponded to a diacetyl dibenzoyl methylhexoside.

4.170 mg. substance: 9.415 mg. CO_2 and 2.070 mg. H_2O
5.875   "   "   : 2.831 " AgI
          $C_{25}H_{26}O_{10}$. Calculated. C 61.70, H 5.39, OCH_3 6.38
          486.2   Found.   " 61.56, " 5.55,   "   6.36

The substance, on heating, sintered at 123° and melted at 131–132°. Its rotation, in chloroform, was $[\alpha]_D^{20} = \dfrac{+3.19° \times 100}{2 \times 2.0} =$ +79.8°. Josephson[9] reported 166° as the melting point of the product which he suggested to be 2,3-dibenzoyl-4,6-diacetyl-β-methylglucoside.

2,3,6-Tribenzoyl-β-Methylglucoside—The syrupy product from the hydrolysis of 10 gm. of the dibenzoyl-benzylidene-β-methylglucoside was dissolved in 60 cc. of dry pyridine and the solution was stirred mechanically and cooled in running water. A solution of 3.2 cc. (1.4 mols per mol) of benzoyl chloride diluted to 18 cc. volume with chloroform, was added slowly over a period of about 20 minutes. The mixture was allowed to stand at room temperature for 4 hours and 5 cc. of water were added. After standing another half hour, ice water and chloroform were added and the extracts were treated as for the substance described above. The washed, dried chloroform extracts were concentrated under reduced pressure to a thick syrup and this was dissolved in a little warm ether. On cooling this solution, the product immediately crystallized. Yield 9 gm. The product was recrystallized by dissolving in warm ether, cooling, and adding pentane. It also crystallized readily from 75 per cent alcohol. The composition corresponded to a tribenzoyl methylhexoside.

4.349 mg. substance: 10.610 mg. CO_2 and 2.090 mg. H_2O
6.845   "   "   : 3.031 " AgI
          $C_{28}H_{26}O_9$. Calculated. C 66.38, H 5.18, OCH_3 6.12
          506.2   Found.   " 66.52, " 5.37,   "   5.84

The melting point was 144.5–145.5° and the rotation, in chloroform, was $[\alpha]_D^{20} = \dfrac{+3.28° \times 100}{2 \times 2.0} = +82.0°$.

2,3,4-Tribenzoyl-β-methylglucoside has been described, but only as a syrup,[10] and no rotation was reported. 2,3,4,6-Tetrabenzoyl-β-methylglucoside[11] melts at 160–162° and has a rotation, in chloroform, of $[\alpha]_D^{20} = +30.99°$.

4-Acetyl-2,3,6-Tribenzoyl-β-Methylglucoside—2 gm. of the tribenzoyl-β-methylglucoside were dissolved in 10 cc. of dry pyridine and 0.6 cc. (1½ mols) of acetic anhydride were added. The mixture warmed slightly. After standing 24 hours at room temperature, 2 cc. of water were added. After ½ hour, more water was added and the mixture was three times extracted with chloroform, and worked up as for the tribenzoyl compound above. After concentrating the washed, dried extract to a thick syrup, ether was poured into the flask and the product crystallized instantly. The ether was evaporated off and the product was recrystallized from methyl alcohol. After recrystallization from the same solvent, the yield was 1.2 gm. and an additional 0.6 gm. of less pure material was recovered from the mother liquors.

The composition of the pure product corresponded to an acetyl tribenzoyl methylhexoside.

4.653 mg. substance: 11.235 mg. CO_2 and 2.090 mg. H_2O
6.511 "   "   : 2.885 " AgI
$C_{30}H_{28}O_{10}$. Calculated. C 65.67, H 5.15, OCH_3 5.66
548.2   Found.   " 65.84, " 5.02,   " 5.85

The melting point was 156–157° and the rotation, in chloroform, was $[\alpha]_D^{20} = \dfrac{+3.84° \times 100}{2 \times 2.0} = +96.0°$.

6-Acetyl-2,3,4-tribenzoyl-β-methylglucoside has been described.[10,12] The melting point is 150–151° and the rotation, in chloroform, is $[\alpha]_D^{20} = -6.5°$.

4-p-Tolylsulfo-2,3,6-Tribenzoyl-β-Methylglucoside—10 gm. of pure tribenzoyl-β-methylglucoside were dissolved in 50 cc. of dry pyridine, 4.1 gm. (1.1 mols) of p-toluenesulfonchloride were added, the mixture was shaken until the chloride dissolved and was then kept at 50° for 24 hours. Water was added just to turbidity, and after ½ hour at room temperature, more water was added. The

[10] Josephson, K., *Ber. chem. Ges.*, **62**, 313 (1929).

[11] Fischer, E., and Helferich, B., *Ann. Chem.*, **383**, 90 (1911).

[12] Bergmann, M., and Koch, F. K. V., *Ber. chem. Ges.*, **62**, 311 (1929).

mixture was extracted three times with chloroform and the extracts were worked up as for the compounds above. The washed, dried extracts were concentrated to a syrup which crystallized immediately upon addition of ether. From the ether solution additional material was recovered, making the total yield of crude product 6.6 gm. It was recrystallized by dissolving in warm ethyl acetate, adding an equal volume of hexane, and cooling. After 2 hours in the ice box, the crystals were filtered off, washed with ethyl acetate-hexane, and air-dried at 50°. Material twice crystallized in this manner had a composition which corresponded to a tolyl-sulfo tribenzoyl methylhexoside.

4.900 mg. substance: 11.475 mg. CO₂ and 2.218 mg. H₂O

$$\text{4.900 mg. substance: 11.475 mg. } CO_2 \text{ and 2.218 mg. } H_2O$$

0.1530 gm.   "   : 0.0576 gm. AgI

13.640 mg.   "   : 4.699 mg. BaSO₄

$C_{35}H_{32}O_{11}S$. Calculated. C 63.60, H 4.92, OCH_3 4.69, S 4.85

660.3   Found.   " 63.87, " 5.06, " 4.96, " 4.74

The product melted and decomposed at 191–192° and had a rotation, in chloroform, of $[\alpha]_D^{20} = \dfrac{+1.72° \times 100}{2 \times 2.0} = +43.0°$.

Experiments designed to remove the benzoyl groups, while leaving the tolylsulfo group intact, have thus far been unsuccessful.

4-Methyl-2,3,6-Tribenzoyl-β-Methylglucoside—10 gm. of the pure tribenzoyl-β-methylglucoside were dissolved in 40 cc. of methyl iodide and methylated by the Purdie method, 16 gm. of silver oxide being added in four equal portions at ½ hour intervals. After the methylation, the product was isolated by extraction with boiling chloroform, and the filtered extracts were concentrated under reduced pressure to a syrup. This was redissolved in methyl iodide and remethylated exactly as before. The final product was a syrup which did not crystallize. A small portion was dried to a solid transparent mass over phosphorus pentoxide, powdered, and redried under greatly reduced pressure. The analysis indicated it to be incompletely methylated.

6.321 mg. substance: 5.251 mg. AgI

$C_{29}H_{28}O_9$. Calculated. OCH_3 11.92

520.2   Found.   " 10.96

The rotation, in chloroform, was $[\alpha]_D^{21} = \dfrac{+2.34° \times 100}{2 \times 2.0} = +58.5°$.

4-Methyl-2,3,6-Triacetyl-β-Methylglucoside—The syrup obtained above was dissolved in 50 cc. of methyl alcohol. 0.3 cc. of a 2.4 N solution of barium oxide in methyl alcohol was added and the mixture was kept at room temperature for 1 hour and then at 50° for 1 hour. An additional 0.3 cc. of the barium oxide solution was added and the mixture was refluxed on the steam bath for an hour. It was then concentrated under reduced pressure to about 10 cc. volume, ether was added, and the flocculent precipitate which formed was filtered off and discarded. The filtrate was concentrated under reduced pressure to a syrup which was dissolved in 75 cc. of pyridine. 6.5 cc. of acetic anhydride (3.3 mols) were added and the mixture was allowed to stand overnight at room temperature. Water was added and after $\frac{1}{2}$ hour the product was isolated by chloroform extraction and worked up as for the substances above. After concentrating the washed, dried chloroform extract to a thick syrup, it was dissolved in ether and cooled. Pentane was added just to turbidity, and the mixture was allowed to stand in the ice box. The product crystallized after 2 days. Yield 2.6 gm. It was recrystallized once from ether by adding pentane, and then once from ether alone. The composition corresponded to a methyl triacetyl methylhexoside.

4.105 mg. substance: 7.594 mg. CO_2 and 2.501 mg. H_2O
4.550 " " : 6.435 " AgI
$C_{14}H_{22}O_9$. Calculated. C 50.27, H 6.63, OCH_3 18.55
334.2 Found. " 50.44, " 6.81, " 18.66

The rotation, in chloroform, was $[\alpha]_D^{20} = \dfrac{-1.31° \times 100}{2 \times 2.0} =$ −32.8°. The melting point was 107–108° and the melting point of an intimate mixture with the 4-methyl triacetyl-β-methylglucoside described below was the same, 107–108°.

4-Methyl-2,3,6-Triacetyl-β-Methylglucoside—The "trimethyl" dibenzylmercaptoglucose of Pacsu[2] was twice recrystallized. The methoxyl content (7.25 per cent) agreed with Schinle's[1] conclusion that the substance is a monomethyl dibenzylmercaptoglucose (methoxyl 7.31 per cent). It was hydrolyzed as described by Pacsu to the free methyl glucose, obtained as a syrup. This was converted to the tetracetate with pyridine and acetic anhydride and the syrupy product was converted to the acetobromo

compound with a saturated solution of hydrogen bromide in glacial acetic acid in the usual manner. No attempt was made to crystallize the bromo compound; instead, it was dissolved in dry methyl alcohol and shaken with dry silver oxide until the test for halide was negative. The mixture was filtered with charcoal and concentrated under reduced pressure to a small volume. The product crystallized on standing overnight in the ice box. It was filtered off, washed with a little methyl alcohol, and dried. For recrystallization it was dissolved in ether and cooled, and pentane was added just to turbidity. After standing overnight, the substance was filtered off, washed with the same solvents, and recrystallized from ether. The product thus obtained was air-dried. The composition corresponded to a methyl triacetyl methylhexoside.

4.765 mg. substance: 8.830 mg. CO_2 and 2.800 mg. H_2O
3.115 " " : 4.301 " AgI
$\quad C_{14}H_{22}O_9$. Calculated. C 50.27, H 6.63, OCH_3 18.55
$\quad$ 334.2 $\quad$ Found. " 50.53, " 6.57, " 18.22

The rotation, in chloroform, was $[\alpha]_D^{20} = \dfrac{-1.34° \times 100}{2 \times 2.0} = -33.5°$.

The melting point was 107–108° and a mixed melting point with the methyl triacetyl methylglucoside described above was the same, 107–108°.

2,3-Diacetyl-β-Methylglucoside—Diacetyl benzylidene-β-methylglucoside was prepared by acetylation of benzylidene-β-methylglucoside with acetic anhydride and sodium acetate in the usual manner. It was then deacetylated in a manner similar to that described for the corresponding benzoyl compound. 0.025 N hydrochloric acid, as used by Oldham and Rutherford[6] was found to give better results than the 0.1 N which we used initially. The hydrolyzing mixture was refluxed for 5 hours, however, instead of 4. For isolation, the hydrochloric acid was removed by shaking the cooled solution with silver oxide. The mixture was filtered and the filtrate was concentrated under reduced pressure until two layers formed, extracted three times with pentane in a separatory funnel, and filtered with charcoal. The filtrate was concentrated under reduced pressure to a thick syrup.

2,3-Diacetyl-4,6-Dibenzoyl-β-Methylglucoside—The syrup, obtained as above from 8.2 gm. of diacetyl benzylidene-β-methyl-

glucoside was distilled with pyridine and benzene for some time to remove all water and then dissolved in 50 cc. of dry pyridine. 7.7 cc. (3 mols) of benzoyl chloride were added and the mixture was allowed to stand overnight at room temperature. 5 cc. of water were added, and after $\frac{1}{2}$ hour, ice water and chloroform. The product was extracted three times with chloroform and worked up as described for the derivatives above. The washed, dried chloroform extracts were concentrated under reduced pressure to a syrup, and ether was added. Crystallization started immediately. After 3 or 4 hours in the ice box, the product was filtered off, washed with ether, and dried. The yield was 6.1 gm. and an additional 1.6 gm. was obtained by working up the mother liquor. The product was three times recrystallized from 10 to 12 parts of absolute alcohol. Its composition corresponded to a diacetyl dibenzoyl methylhexoside.

4.476 mg. substance: 10.115 mg. CO_2 and 2.200 mg. H_2O
6.187 "   "   : 3.050 " AgI
$\quad\quad$ $C_{25}H_{26}O_{10}$. Calculated. C 61.70, H 5.39, OCH_3 6.38
$\quad\quad$ 486.2   Found.   " 61.63, " 5.50, " 6.51

The substance melted at 164–165° and had a rotation, in chloroform of $[\alpha]_D^{20} = \dfrac{-0.23° \times 100}{2 \times 2.0} = -5.8°$.

2,3,6-Triacetyl-β-Methylglucoside—The syrup from the hydrolysis of 25 gm. of diacetyl benzylidene-β-methylglucoside was distilled with benzene and pyridine to remove remaining water and was then dissolved in 50 cc. of dry pyridine. The solution was stirred mechanically and cooled in running water. A solution of 7.5 cc. of acetic anhydride (1.1 mols) in 30 cc. of chloroform was added drop by drop over a period of about 20 minutes. The mixture was then allowed to stand overnight at room temperature and 5 cc. of water were added. After $\frac{1}{2}$ hour the product was isolated with chloroform as described above. The washed, dried chloroform extracts were concentrated under reduced pressure to a thick syrup which was dissolved in a little warm ether. On standing in the ice box for a few hours, crystals appeared, and on stirring with a rod, the product crystallized promptly. It was filtered off, washed with ether, and dried. Yield 11 gm. Further amounts were obtained from the mother liquors only with difficulty.

The product was dissolved in boiling ether and concentrated to about 50 cc. The solution was cooled, an equal volume of pentane was added, and the product immediately crystallized. It was then twice recrystallized by dissolving in boiling ether, concentrating, and cooling. The composition of the product corresponded to a triacetyl methylhexoside.

4.799 mg. substance: 8.557 mg. CO_2 and 2.741 mg. H_2O
4.751 "   "   : 3.580 " AgI
    $C_{13}H_{20}O_9$. Calculated. C 48;70, H 6.29, OCH_3 9.68
    320.2   Found.   " 48.62, " 6.38,   " 9.94

In an acetyl determination, 0.100 gm. required 9.37 cc. of 0.1 N sodium hydroxide, while the theoretical value is 9.44 cc.

The melting point of the pure product was 113.5–114.5° and the rotation, in chloroform, was $[\alpha]_D^{20} = \dfrac{-2.36° \times 100}{2 \times 2.0} = -59.0°$. Helferich, Bredereck, and Schneidmüller[4] give 114–115° as the melting point of their 2,3,6-triacetyl-β-methylglucoside and −64.9° as its rotation in chloroform. 2,3,4-Triacetyl-β-methylglucoside[4] melts at 134° and has a rotation, in chloroform, of −18.8°.

4-Benzoyl-2,3,6-Triacetyl-β-Methylglucoside—1.5 gm. of the triacetyl-β-methylglucoside, obtained above, were dissolved in 15 cc. of dry pyridine, and 1.1 cc. of benzoyl chloride (2.2 mols) were added. After standing overnight at room temperature, 2 cc. of water were added, and after another half hour, more water. The product was isolated by chloroform extraction and worked up as for the products above. The washed, dried chloroform extract was concentrated under reduced pressure to a thick syrup which was dissolved in ether. After cooling, pentane was added just to turbidity. After a few days in the ice box, a few crystals formed, and on scratching with a glass rod the product crystallized immediately. The total yield was 1.7 gm. The product was crystallized from ether by adding pentane, and then once from ether alone. The composition corresponded to a benzoyl triacetyl methylhexoside.

5.005 mg. substance: 10.395 mg. CO_2 and 2.520 mg. H_2O
6.085 "   "   : 3.358 " AgI .
    $C_{20}H_{24}O_{10}$. Calculated. C 56.58, H 5.70, OCH_3 7.31
    424.2   Found.   " 56.63, " 5.63,   " 7.28

The melting point was 98–99° and the rotation, in chloroform, was $[\alpha]_D^{20} = \dfrac{-2.44° \times 100}{2 \times 2.0} = -61.0°$.

Ohle and Spencker[13] have described 6-benzoyl-2,3,4-triacetyl-β-methylglucoside, melting point 127° and rotation $+15.15°$.

4-p-Tolylsulfo-2,3,6-Triacetyl-β-Methylglucoside—1.5 gm. of the triacetyl-β-methylglucoside were dissolved in 15 cc. of dry pyridine and 1.8 gm. (2 mols) of p-toluenesulfonchloride were added. The mixture was shaken until all the chloride dissolved and was then allowed to stand at room temperature overnight. 2 cc. of water were added, and after $\frac{1}{2}$ hour, more water. The product was extracted with chloroform and worked up as described for the compounds above. The washed, dried chloroform extract was concentrated under reduced pressure to a thick syrup, which was dissolved in a little ether. On adding pentane to turbidity, the product crystallized immediately. The first crop weighed 1.6 gm. and the mother liquors were not worked up for further quantities. It was twice recrystallized from absolute alcohol. The composition corresponded to a tolylsulfo triacetyl methylhexoside.

4.821 mg. substance: 9.007 mg. CO_2 and 2.290 mg. H_2O
4.792 "   "   : 2.455 " AgI
11.880 "   "   : 5.702 " $BaSO_4$
$C_{20}H_{26}O_{11}S$. Calculated. C 50.61, H 5.53, OCH_3 6.54, S 6.76
474.2   Found.   " 50.94, " 5.31, "   6.76, " 6.59

The melting point was 116–117° and the rotation in chloroform was $[\alpha]_D^{20} = \dfrac{-1.60° \times 100}{2 \times 2.0} = -40.0°$ and in pyridine $[\alpha]_D^{20} = \dfrac{-1.18° \times 100}{2 \times 2.0} = -29.5°$. Helferich, Bredereck, and Schneidmüller[4] give 118° as the melting point, and $-29.7°$ as the rotation in pyridine, of the p-tolylsulfo derivative of their 2,3,6-triacetyl-β-methylglucoside. The 6-p-tolylsulfo-2,3,4-triacetyl-β-methylglucoside[4,13] has a melting point of 171° and rotations in pyridine and chloroform respectively of $+33.1°$ and $+12.03°$.

[13] Ohle, H., and Spencker, K., *Ber. chem. Ges.*, **59**, 1836 (1926).

Addendum—While this paper was in press we found a very recent article by Brigl and Grüner (*Ann. Chem.*, **495,** 60 (1932)) in which 2,3-dibenzoyl-4,6-diacetyl-β-methylglucoside was prepared by the same method which we employed. Almost identical properties were reported (m. p. 132–133° and $[\alpha]_D^{20} = +79.3$ CHCl₃). These authors assign a structure of 2,6-dibenzoyl to the compounds of Josephson.

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PROCEEDINGS OF THE AMERICAN SOCIETY OF BIOLOGICAL CHEMISTS

Twenty-Sixth Annual Meeting

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OBSERVATIONS ON GROWTH FROM THE VIEW-POINT OF STATISTICAL INTERPRETATION

By H. C. SHERMAN AND H. L. CAMPBELL

(*From the Department of Chemistry, Columbia University, New York*)

Frequency diagrams for relatively large numbers of (1) weights of experimental animals (rats) at 28 days of age, and (2) gains in weight during the 5th to 8th weeks of age inclusive are shown as illustrating the degree in which such data may be expected to approximate the symmetrical distribution which is assumed as the basis of the usual statistical interpretation.

CHEMICAL CHANGES IN THE BLOOD FOLLOWING MAGNESIUM DEPRIVATION

By H. D. KRUSE, ELSA ORENT,* AND E. V. McCOLLUM

(*From the Biochemical Laboratory, School of Hygiene and Public Health, the Johns Hopkins University, Baltimore*)

When young rats and dogs are restricted to a diet which contains only 1.8 parts per million of magnesium but is otherwise adequate, they soon show a succession of symptoms; namely, vasodilatation, spasticity of muscles, and tonic-clonic convulsions terminating in death. Earlier we presented the view that this condition represents tetany, and that vasodilatation gives it distinction from other forms of tetany. At that time we suggested also that tetany is inducible by a variety of means, that lowered blood magnesium should theoretically lead to the condition, and that slight variations in the symptomatic pattern might be expected, dependent upon the method of producing the syndrome. It was realized then that blood studies would be necessary to demonstrate the differential character of tetany produced by a restricted magnesium intake.

In the present communication we are prepared to report the chemical changes in the blood of ten dogs deprived of magnesium.

Weekly determinations throughout the period of magnesium deprivation were made on the following blood constituents: Na, K, Ca, Mg, P, fatty acids, total cholesterol, cholesterol esters, lipoid P, bilirubin, CO_2-combining power, pH, Cl, fibrinogen, albumin, globulin, non-protein N, sugar, creatine, creatinine, and volume percentage of erythrocytes. The significant changes are: an early and progressive decrease in magnesium; several weeks prior to death a persistent rise in total cholesterol due to an increase in cholesterol esters; shortly thereafter a fall in the volume percentage of erythrocytes; terminally a slight elevation in sugar, and a rise in non-protein nitrogen due largely but not entirely to an increase in creatine. The other blood constituents remain unchanged. Control animals, receiving adequate magnesium, show values for all constituents within normal limits.

The fact that the CO_2-combining power is undisturbed in the face of an extremely low blood magnesium indicates that either the latter element plays little part in acid-base equilibrium or its function can be readily compensated. What is most important is that tetany is shown to occur with normal blood calcium values. The results appear to support the view that lowered magnesium in the blood is capable of inducing tetany independent of any changes in the calcium level.

CHEMICAL STUDIES OF THE SUPRARENAL GLAND

By EDWARD C. KENDALL

(From the Division of Chemistry, The Mayo Foundation, Rochester, Minnesota)

Lactic acid is found in a higher concentration in the suprarenal gland than any other part of the animal organism. It appears probable that epinephrine is retained in the gland as the lactate salt. If the suprarenal gland is ground while in a frozen condition and dropped into cold acetone, practically all of the epinephrine, hexuronic acid, and lactic acid are extracted by the acetone within 24 hours. The residue is then pressed and the acetone concentrated to a small volume. Such a solution contains only traces of fat and lecithin. If sodium bicarbonate is added to the solution and it is then extracted with ether, a derivative of epinephrine is found dissolved in the ether. It may be extracted from the ether

with water. Investigation has shown this derivative to contain lactic acid. It therefore seemed probable that the derivative was lactyl epinephrine. Whether the lactic acid radical is attached to the methylamine group or the secondary alcohol group of the side chain, has not been definitely determined. The two phenolic groups are free. The compound easily reduces sodium phosphotungstate and is oxidized by ferricyanide at pH 8. It gives the characteristic color with ferric salts which is identical with the color given by epinephrine. The lactic acid is easily oxidized, particularly in a solution of ether. The oxidized product appears to be the pyruvic derivative, since an oxidized solution contains more lactic acid after reduction than before. The lactyl derivative of epinephrine has been prepared synthetically from methyl lactate and epinephrine. It possesses the same solubility and chemical properties as the product separated from the gland.

The significance of this derivative has not been fully established. It is possible that it is involved as an intermediate compound in the reaction discovered by Cannon which results in the liberation of sympathin. Injection of the compound does not cause blanching of the mucous membrane or the response which is characteristic of epinephrine. No method has been found to liberate the epinephrine from the derivative by hydrolysis. This suggests that in the animal organism, the epinephrine is liberated by oxidation of the derivative rather than by hydrolysis.

Both the natural product and the synthetic lactyl epinephrine have a marked effect on restoration of a dog in prostration after complete suprarenalectomy, but they cannot indefinitely prolong the life of such an animal.

THE QUANTITATIVE APPLICATION OF THE ANTIMONY TRICHLO-RIDE COLOR REACTION FOR VITAMIN A

By HARRY E. DUBIN AND C. W. HOOPER

(From the H. A. Metz Laboratories, Inc., New York)

The color test is carried out according to the method of Norris and Church. Varying concentrations of the non-saponifiable fraction of cod liver oil are used. The results, in terms of blue units, are plotted. From the curves obtained it is possible to figure, within 10 per cent, the vitamin A potency of an unknown

sample.   The results obtained by this method are in substantial
agreement with the biological assay.

THE RATE OF TRANSFER OF INGESTED VITAMIN A TO THE LIVER OF THE RAT

By E. M. NELSON, REED WALKER, and D. BREESE JONES

(From the Protein and Nutrition Division, Bureau of Chemistry and Soils,
United States Department of Agriculture, Washington)

The technique of determining vitamin A described by the
authors a year ago, whereby single doses of vitamin A-containing
material are fed to animals on a vitamin A-deficient ration, has
been applied to studying the storage of vitamin A.

When given doses of cod liver oil are fed to the rat, a definite
percentage of vitamin A is transferred to the liver and this transfer
is completed within 24 hours from the time of ingestion of the oil.

The technique of vitamin A determination used in these studies
has proved to be a simple and accurate method of investigating
the storage of vitamin A in the liver of the rat.

FURTHER STUDIES ON THE SPECIFIC EFFECT OF VITAMIN B ON GROWTH AND ON LIPID METABOLISM IN AVITAMINOSIS

By BARNETT SURE, M. C. KIK, and ANNA E. CHURCH

(From the Departments of Agricultural Chemistry and Home Economics,
University of Arkansas, Fayetteville)

Our studies on the *specific* effect of vitamin B on growth were
continued during the last year with highly concentrated vitamin B
preparations, with as little as 2 to 3 mg. daily as a source of the
vitamin B complex, and as little as 1 mg. daily as a source of
vitamin B (B_1), and all of our results conclusively substantiate
our previous findings that vitamin B (B_1) *per se* influences growth
unrelated to the plane of nutrition.   However, our further investi-
gations on lipid metabolism in avitaminosis do not confirm our
preliminary findings that lipemia is a symptom complex of vitamin
B deficiency.   In weaned rats the variations are so great in the
individual determinations of fatty acids and cholesterol of the
blood of control and pathological animals that, after a statistical
treatment of the data, we find no influence of this avitaminosis
on the concentration of the lipid constituents in the blood.   Our

previous conclusion of lipemia in polyneuritic nursing young is due to suckling and not to vitamin B; in other words, what we encountered was probably nothing more than an alimentary lipemia, since the lactating albino rat secretes milk with a fat content of about 30 per cent. Our preliminary work to date on vitamin A deficiency shows a reduction in the concentration of fatty acids in the blood during advanced stages associated with marked ophthalmia and preceding total collapse.

THE EFFECT OF VITAMINS B₁ AND B₂ ON THE APPETITE AND THE UTILIZATION OF FOOD IN RATS

By WENDELL H. GRIFFITH AND CLAIRE E. GRAHAM

(*From the Department of Biological Chemistry, St. Louis University School of Medicine, St. Louis*)

The relation of vitamins B_1 and B_2 to the consumption and utilization of food was studied in over 500 rats. Young males, 30 days of age, were fed the Evans and Burr diet (purified casein 25, sucrose 75, salt mixture 4) supplemented with cod liver oil and one or more of the following: yeast (vitamins B_1 and B_2), tikitiki (vitamin B_1), or autoclaved liver (vitamin B_2). All of the rats were restricted to either 975 or 1365 calories during a 40 day experimental period.

If adequate vitamin B_1 was fed to groups of rats receiving decreasing quantities of vitamin B_2 appetite was found to fail before the efficiency of utilization of food was affected. The results were the same if adequate vitamin B_2 was fed to groups of rats receiving decreasing quantities of vitamin B_1. If both vitamins B_1 and B_2 were decreased, utilization of food was affected before appetite failed. The experiments suggested a close relationship between vitamins B_1 and B_2 and those metabolic processes controlling appetite and the utilization of food.

THE EFFECT OF ULTRA-VIOLET RAYS ON THE DERMATITIS-PREVENTING VITAMIN

By ALBERT G. HOGAN AND LUTHER R. RICHARDSON

(*From the Departments of Animal Husbandry and Agricultural Chemistry, University of Missouri, Columbia*)

A few days after birth the experimental animals are transferred to a cage with a hardware cloth bottom. At the age of 17 days

they are given the experimental diet, and are weaned at 21 to 23 days, depending on their weights, when they are placed in individual cages. After a depletion period of approximately 7 days, depending on the vigor of the rats, the feeding of the yeast supplements is begun, at a level of 100 mg. daily. Irradiation for a period of 10 hours has proved sufficient, though a longer period may be necessary if an old lamp is used.

The ration consists of casein 23, sucrose 68, cellulose 3, a mineral mixture 4, and cod liver oil 2. The casein is leached with acid water, extracted with alcohol and ether, and the sucrose is recrystallized. These are mixed with the cellulose and mineral mixture, moistened, heated in a vessel of boiling water for 4 hours, dried, and ground. The cod liver oil is then added. It may prove possible further to improve the ration, and some of the precautions may be unnecessary. Of eighteen rats subjected to this, or a slightly modified régime, seventeen have developed a definite dermatitis, and the other is diagnosed as showing the early symptoms.

In a few cases a definite diagnosis of dermatitis was made at 52 days of age; in others, when the rats were approximately 9 weeks old. Insufficient time has elapsed to determine the survival period. Our data do not show whether vitamin G is completely destroyed, or to what extent vitamin B may be affected.

FACTORS WHICH INFLUENCE THE EFFECTIVENESS OF A RACHITOGENIC RATION

By ARTHUR D. HOLMES AND FRANCIS TRIPP

(*From the Research Laboratories, The E. L. Patch Company, Boston*)

Analyses of Steenbock's Ration 2965 prepared under normal laboratory conditions show a lack of uniformity in the ash, calcium, and phosphorus content and hence a lack of uniformity in the ratio of calcium to phosphorus. Since yellow corn constitutes the major portion of this ration, samples were secured from the corn-producing areas of the United States. When these were analyzed it was found that their ash, calcium, and phosphorus content varied materially. These findings are in accord with similar studies reported by several investigators. The reported results of studies of the protein content of samples of corn grown under identical conditions indicate that this constituent may vary over wide limits. A number of investigators have determined the vitamin A content of yellow corn grown in widely distributed areas. Their results show that this factor is decidedly variable. In fact, they found that the vitamin A content not only varied with different varieties of corn, but also varied significantly for the same variety of corn when grown on the same soil during successive years. While relatively little attention has been given to the vitamin D content of yellow corn, one group of investigators maintains that this factor varies enough to affect the development of experimental rickets. It is evident from the foregoing that different lots of yellow corn as obtained in the open market are not uniform as regards ash, calcium, phosphorus, protein, vitamin A, and vitamin D content. Pronounced variability of any of these factors influences the effectiveness of the rachitogenic ration. From these observations it is concluded that the rachitogenic ration under discussion is excellent for qualitative vitamin D studies, that it is valuable for isolated quantitative vitamin D determinations, but it is felt that when a laboratory proposes to determine the vitamin D content of a series of products for comparative purposes or when two or more laboratories wish to conduct a simultaneous assay of the vitamin D content of some product, definite attention should be given to those factors which influence the effectiveness of the ration.

DOES THE RATIO OF CALCIUM TO PHOSPHORUS OF THE DIET DETERMINE WHETHER RICKETS IS PRODUCED IN THE RAT?

By ALFRED T. SHOHL, HELEN BENNETT BROWN, CATHERINE S. ROSE, AND ESTHER SAUERWEIN

(From the Babies' and Children's Hospital and the Department of Pediatrics, School of Medicine, Western Reserve University, Cleveland)

The high calcium-low phosphorus type of diet regularly produces rickets when the calcium and phosphorus are at the level of those in the McCollum and Steenbock diets. When the level of both calcium and phosphorus is raised or lowered (the ratio between them being kept the same) the rickets-producing quality of the diet is diminished or increased respectively. Thus at each level of calcium a different ratio is necessary to produce rickets, as measured by x-ray, histology, blood serum calcium and phosphorus, and ash of the bones.

THE PREPARATION AND PROPERTIES OF A CONCENTRATE OF VITAMIN E FROM LETTUCE

By H. S. OLCOTT

(From the Laboratory of Biochemistry, State University of Iowa, Iowa City, and the Laboratory of Physiological Chemistry, Yale University, New Haven)

A preparation of vitamin E was obtained from lettuce by fractional crystallization and separation of the unsaponifiable lipids. The method used was essentially that of Evans and Burr,[1] and has been described before.[2] This material was still further concentrated by fractional distillation *in vacuo*. The fraction collected from 190–220° (0.1 mm.) was the most active. After the removal of traces of sterols and other solid alcohols by recrystallization from acetone, 10 mg. were sufficient, when fed to a female rat deficient in vitamin E, to allow the birth of a normal litter. Concentrates so obtained were resin-like materials which resisted crystallization. Some of the physical and chemical properties were determined. Neither acetylation nor hydrogenation destroyed the activity.

[1] Evans, H. M., and Burr, G. O., Memoirs of the University of California, Berkeley, **8** (1927).

[2] Olcott, H. S., and Mattill, H. A., *J. Biol. Chem.*, **93**, 59 (1931).

ON A METHOD WHEREBY THE PRINCIPLE WHICH IS EFFECTIVE IN THE TREATMENT OF PERNICIOUS ANEMIA MAY BE OBTAINED FROM LIVER IN SUBSTANTIALLY LARGER AMOUNT

By GEORGE B. WALDEN AND G. H. A. CLOWES

(From The Lilly Research Laboratories, Eli Lilly and Company, Indianapolis)

Since Minot's discovery of the efficacy of raw liver in the treatment of pernicious anemia, numerous extracts and concentrates of liver have been prepared, but the production of such liver preparations has always been associated with a loss of potency. In the best of these extracts (the Cohn Fraction G, now most commonly employed) approximately 75 per cent of the original activity of the raw liver is recovered.

Castle's observation that beef muscle, itself inert, when digested with normal human stomach juice, also in itself inert, yielded an active product, suggested the possibility of increasing the yield of the active principle obtainable from raw liver by interaction with stomach tissues or extracts.

We have now demonstrated experimentally that the therapeutic efficacy of raw liver or of the Cohn liver Fractions D and G may be increased at least 3- or 4-fold by interaction or digestion with small amounts of stomach tissues under appropriate physical and chemical conditions. These preparations were tested on a series of pernicious anemia cases in relapse, the reticulocyte peaks, increases in red blood cell count, and general improvement of the patients affording a basis of comparison with results obtained on comparable cases using fresh raw liver and Liver Extract 343.

THE HEMOGLOBIN CONTENT OF THE BLOOD OF INFANTS

By C. A. ELVEHJEM AND W. H. PETERSON

(From the Department of Agricultural Chemistry, University of Wisconsin, Madison)

The hemoglobin content of the blood of a large number of children was determined in order to establish normal values for average healthy children. This study was made possible through the cooperation of Dr. Dorothy Reed Mendenhall, Chairman of the Child Health Centers of the Madison Public Health Nursing Associations. The determinations were made on the children brought to these clinics during the past 4 years.

The samples were taken between the hours of 2 and 5 in the afternoon and the interval between determinations on the same child was 2 to 4 weeks. Blood was obtained from the toe and the amount of hemoglobin determined by the Newcomer method. A total of over 2000 readings was made on about 700 individual children, varying in age from birth to 5 years.

The hemoglobin content of the blood of the infants was averaged for each month of age. All values below 8.5 gm. of hemoglobin per 100 cc. of blood were not included in these averages. These data show that the hemoglobin content of the blood is exceedingly high at birth (above 20 gm. per 100 cc. of blood) and falls very rapidly during the first 2 months of life to values of 12 to 13 gm. The average figures remain between 11 and 13 gm. until the age of 2½ years is reached. The values are lowest at about 15 months, after which there is a gradual increase to values of 13 to 14 gm. at 3 to 4 years of age. A tabulation of the highest and lowest values for each period shows that there are great variations in the amount of hemoglobin in the blood of children brought to the average clinic. The average figures are somewhat lower than those obtained for individual children on controlled diets.

DIET AND HEMORRHAGIC DISEASE

By I. NEWTON KUGELMASS

(*From the Department of Pediatric Research, Fifth Avenue Hospital, New York*)

Dietary factors alter the hemorrhagic status of the blood and the permeability of the vascular endothelium. The results are a tendency to either bleeding or clotting. The hemorrhagic status was evaluated by determining quantitatively the concentration of the blood-clotting components—prothrombin, fibrinogen, antithrombin, platelets, and degree of platelet lysis—and the status of the vascular endothelium was determined by capillary resistance tests.

Potential and active hemorrhagic disease is the result of either a change in the concentration of the blood-clotting components or an alteration in the vascular endothelium. The ratio of the concentration of substances tending towards clotting over those tending towards bleeding has been evaluated as the index of blood-clotting function in health and in disease. Experimental studies have been initially made on rats, guinea pigs, and dogs to deter-

mine quantitatively the degree of bleeding and clotting produced in the animal mechanism. The results show that prolonged feeding of diets containing protein markedly increases the coagulability of the blood in that the prothrombin and fibrinogen rise to higher levels. Dietary protein has been found to affect the mechanism continually in contrast to the introduction of protein into the vascular system which produces a transient effect. Dietary fat abundant in vitamin D elevates blood platelets. Excessive intake of vitamins A, B, and C produces no change in the blood-clotting components but exclusion of these vitamins from the dietary produces a gradual diminution in the fibrinogen content of the blood paralleling the duration of the deficiency. Deficiency in vitamin C, of course, produces changes in the endothelium without any alteration in the concentration of the blood-clotting components. Excessive vitamin D in the dietary tends to elevate the blood platelets, while deprivation of the vitamin diminishes the prothrombin and the fibrinogen content of the blood without appreciable change in the platelet concentration.

Acid-forming dietaries and acidosis tend to favor increased coagulability of the blood, whereas base-forming dietaries and alkalosis favor decreased coagulability. Ketogenic dehydrating dietaries tend towards thrombosis, while fluid dietaries favor bleeding. These results have led to the development of a clotting diet that is high in proteins and in fats, on the one hand, and a bleeding diet high in carbohydrate, fruits, and vegetables. These dietaries have been tested in 200 patients with potential hemorrhagic and thrombotic manifestations.

SOME OBSERVATIONS ON NUTRITIONAL ANEMIA

By CHARLES J. STUCKY

(From the Department of Chemistry, New York State Psychiatric Institute and Hospital, New York)

Studies on anemia due to milk diet were conducted on more than 400 rats of Wistar Institute strain. Most of the animals were reared by a modification of the procedure of Elvehjem and Kemmerer.[3] Such anemic rats showed at weaning a greater vitality than animals produced by the original technique.

[3] Elvehjem, C. A., and Kemmerer, A. R., J. Biol. Chem., **93**, 189 (1931).

Four different diets were employed for feeding the breeders, the offspring of which were used for the anemia experiments.

Powdered whole milk (Klim) was fed in the solid form in order to produce anemia. The growth rate of our rats on this diet seems to be greater than that reported in the literature by other investigators.

In the interpretation of our results we found it advisable to take into consideration the total hemoglobin of the animal. This figure was calculated from the blood volume (Donaldson's tables) and the hemoglobin in gm. per cent.

When the breeders were fed adequate diets, their offspring, rendered anemic on the Klim diet, showed no differences in their response to identical supplements.

Our data on hemoglobin regeneration, resulting from the supplementary feeding of iron salts, resemble, in a general way, those of Mitchell and Miller.[4] However, 10 to 20 per cent of our rats failed to show any recovery on the iron supplements. No difference was observed from the addition of glutamic acid to these iron supplements.

Mothers which were fed from the time of their weaning on a low protein diet (9 per cent casein, lettuce) yielded offspring which reacted differently when rendered anemic by the Klim diet (the same batch of Klim as in the preceding experiments). These rats failed to recover when fed iron supplements and died (seven out of eight) within a few weeks. However, in these rats, the addition of glutamic acid to the iron supplements resulted in good hemoglobin regeneration and growth, which compared favorably to the effect of iron plus copper.

The data obtained indicate that the nutritional history of the animal is an important factor not only in the production of the anemia but also in the recovery. This raises the question whether the copper content of the ration and of the body are the only factors involved in the recovery resulting from the feeding of iron in nutritional anemia. It also suggests the possibility that certain organic factors and the constitution of the experimental animal are important considerations in the results which are obtained.

[4] Mitchell, H. S., and Miller, L., *J. Biol. Chem.*, **92**, 421 (1931).

This may help to explain some of the discrepancies in the observations of different investigators.

FACTORS INFLUENCING ANEMIA DEVELOPMENT IN YOUNG RATS

By HELEN S. MITCHELL

(From the Nutrition Research Laboratory, Battle Creek Sanitarium and College, Battle Creek)

Nutritional anemia in young rats is produced in most laboratories by feeding a milk ration from the time of weaning or before. In spite of every precaution that the young shall never obtain any of the mother's dry ration, and shall receive only mother's milk and later cow's milk, the hemoglobin content of the blood of young rats at weaning is far from uniform. The variation is but slight among those of the same sex in any one litter, but there is a difference between sexes and between litters of the same age and heritage which is not easily explained.

Data accumulated in this laboratory on all rats used for anemia work during the past 2 years have been analyzed in an effort to determine what factors, if any, are consistently responsible for the variations noted. Complete records of 570 young rats from 73 litters plus blood histories of the mothers during pregnancy and lactation in forty-two cases afford information which should aid in the standardization of technique to be used in further nutritional anemia work.

The differences in the initial hemoglobin figures of the young at weaning seem to be chiefly responsible for differences in the time required to produce a severe anemia. Correlations have, therefore, been attempted between the initial hemoglobin values of the young rats and several different factors which might conceivably have some influence on this blood story. These considerations are as follows: (1) previous diet of mother; *i.e.*, stock ration with and without mineral and vitamin B reinforcement; (2) parity of the litter; (3) hemoglobin variation of mother's blood during pregnancy; (4) age and weight of the young when first hemoglobin determination was made; (5) size of the litter; (6) method of caging and age when segregated; (7) sex. The last factor has proved most interesting because of a consistently higher

initial hemoglobin value for females than males and a correspond-
ingly longer period required for anemia to develop.

THE ACTION OF COPPER IN IRON METABOLISM

By C. A. ELVEHJEM

(*From the Department of Agricultural Chemistry, University of Wisconsin, Madison*)

The iron content of livers from rats fed iron alone and iron plus
copper for short periods of time was studied in an attempt to
establish the function of copper in hemoglobin formation.

The rats were raised on screens from birth and were allowed to
consume only the mother's milk and whole cow's milk until 21
days of age, when they were placed on cow's milk alone.   The
young were distinctly anemic when 28 to 35 days old.   At weaning,
the total iron content of the rats reared on screens was less than
one-half that of rats allowed to remain on shavings and in contact
with the mother's ration.   Although the percentage of iron in
the rats on screens decreased, the total amount of iron in each rat
increased with age.   The source of this iron must be the mother's
milk.   The iron content of rat milk is at least 3 times as high as
cow's milk.

Livers from anemic rats contained 0.173 mg., those from anemic
rats fed 0.5 mg. of Fe for 14 days contained 0.465 mg., and those
from rats fed 0.5 mg. of Fe for 14 days then 0.05 mg. of Cu alone
for 14 days contained 0.124 mg. of Fe per gm. of dry liver.   The
hemoglobin content of the blood increased during the copper
feeding.

Similar results were obtained when graded levels of iron were
fed with and without copper.   Without copper the storage of iron
in the liver was dependent upon the amount of iron fed.   There
was no increase in the hemoglobin of the blood.   With copper
there was no storage of iron in the liver until the higher levels
of iron were fed, and the increase in the hemoglobin content of the
blood was proportional to the iron intake.

These results demonstrate that in the absence of copper, iron
can be assimilated and stored in the liver.   When copper is fed,
this stored iron can be utilized for hemoglobin building.   When
iron and copper are fed together the iron is used directly for

hemoglobin formation. Copper does not function in the assimilation of iron but acts in the conversion of the iron into forms which can be used for the construction of the hemoglobin molecule.

THE RELATION OF EMMENIN TO OTHER ESTROGENIC HORMONES

By J. B. COLLIP, J. S. L. BROWNE, and D. L. THOMSON

(*From the Department of Biochemistry, McGill University, Montreal, Canada*)

2 years ago physiological studies in placental extracts led us to the conclusion that there was an estrogenic hormone not identical with estrin in the alcohol-soluble ether-insoluble fraction. This conclusion was based on the observation that a dose which was effective in the production of estrus in immature rats (21 days) represented only a small fraction of that required to produce estrus in the recognized test object, the adult ovariectomized rat. Later, one of us prepared a crystalline compound from the ether extract of placenta by the use of the Marrian method.

Physiological studies showed that these crystals had the same properties as the ether-insoluble crude extract. This was reported at the meeting of the American Physiological Society at Montreal, April, 1931, and also published. The crystals were also found to be relatively inactive on immature (21 days) ovariectomized animals. Later, it was shown that the ether-insoluble estrogenic substance of our crude extract could be rendered ether-soluble by autoclaving in 1 per cent acetic acid medium at 150 pounds pressure for 2 hours. It was found also that urine during pregnancy contained an ether-insoluble estrogenic substance similar to that occurring in placental extracts, although the ratio between the effective dose in the immature normal animal and the adult castrated animal was not as great as in the case of material of placental origin. A method of fractionating urine from pregnant animals was devised which allowed of the preparation from the acid-soluble fraction of the ammonium sulfate precipitate of crystalline products which had the same general physiological characteristics as crystalline products obtained from the ether-soluble placental material. Crystals from both sources appear to be identical in appearance and in composition. They are

nearly identical as far as melting point and carbon and hydrogen content are concerned with the trihydroxy estrin of Marrian and Butenandt and the theelol of Doisy.   It is of interest to note that Butenandt has described the preparation of a trihydroxy estrin of low potency and that a similar crystalline preparation of low potency has been prepared from mare urine by Döhrn.   Until physiological studies of a type similar to ours have been made by German workers with their low potency products, the identity of our material with theirs cannot be positively established.

Our best preparations, assayed on the adult ovariectomized rat, show a potency of approximately 60,000 units per gm., but in normal immature animals appear to be as active as a sample of theelol, kindly supplied to us by Dr. Doisy.

Crystalline emmenin has been tested on immature ovariectomized rats; the dose required (10 γ) for a satisfactory response is about 8 times that for the immature normal animal (1.55 γ). There is considerable variation of sensitivity in immature animals. The dose of theelol is about 1½ times greater for a given response when tested on the immature ovariectomized animal than when tested on the immature intact animal.   Transplantation of immature ovaries into the spleen of these immature ovariectomized animals lowers the required amount of both emmenin and theelol to the dosage level of the immature normal animal.   The ovary may contribute part of the estrogenic substance required from its own store or the presence of the ovary in the immature animal may have some influence in increasing the efficiency of utilization of emmenin and to a less degree the efficiency of utilization of theelol.   An increase in the rate of conversion of both emmenin and theelol to some more active form of estrogenic substance might be effective in causing this increased efficiency of utilization.

THE DETERMINATION OF CYSTINE

By HUBERT BRADFORD VICKERY and ABRAHAM WHITE

(From the Biochemical Laboratory of the Connecticut Agricultural Experiment Station and the Laboratory of Physiological Chemistry, Yale University, New Haven)

When proteins are hydrolyzed by boiling with 8 N sulfuric acid in the presence of metallic tin, the cysteine produced can be quan-

titatively precipitated by the addition of an excess of cuprous oxide. The solution is neutralized and an equal volume of alcohol is added; the cuprous mercaptide is centrifuged off and decomposed with hydrogen sulfide in the presence of a little hydrochloric acid. The resulting solution is neutralized to pH 7.2 and aerated until the cysteine is reconverted to cystine; it is then acidified with hydrochloric acid, and the sulfate ion is removed. The cystine content of the solution is calculated from analyses for nitrogen and sulfur; the two results usually agree closely, indicating that little or no nitrogenous substances other than cysteine are precipitated under the conditions adopted. Cystine added to hydrolysates of casein can be recovered to the extent of 93 to 95 per cent, and cystine can be recovered from pure solution to the extent of 98 to 99 per cent. Determinations on a number of proteins by this method have given results of the same order of magnitude as those of Folin and Marenzi.

THE RACEMIZATION AND DECOMPOSITION OF CYSTINE IN ACID SOLUTION

By JAMES C. ANDREWS

(From the Department of Physiological Chemistry, School of Medicine, University of Pennsylvania, Philadelphia)

l-Cystine was dissolved in hydrochloric acid solutions ranging in concentration from 0.5 N to about 6 N (20 per cent constant boiling solution) and also in 2.5 N H_2SO_4, and the solutions were allowed to stand, one series at room temperature and the other at 38° for over 7 years. They were preserved in glass-stoppered bottles but were not protected from the air. Optical activity measurements were made at intervals and, at the end of the experiment, the solutions were tested to determine the degree and type of decomposition which had occurred in addition to the expected racemization.

The results may be summarized as follows:

Racemization of the cystine proceeded, as would be expected, more rapidly in the more concentrated acid and at the higher temperature. A first order velocity constant of fair constancy was produced after the solutions had stood for some weeks and before oxidation had set in to an appreciable extent. However, the solu-

tions racemizing most rapidly not only gave an optical rotation of ultimately zero but continued to become more *dextrorotatory* until an $[\alpha]_D^{25}$ value of about $+10°$ was reached, after which the rotation decreased again towards zero.

After a lapse of about 90 months the HCl solutions of cystine were found to have lost proportions of their original cystine varying from 25 to 100 per cent as determined by the Folin-Marenzi method. Those solutions, which had reached or passed an $[\alpha]$ of 0°, gave negative results with the Folin reagent. This loss in the cystine, as shown by Folin-Marenzi determinations, was confined to the HCl solutions of cystine; in H_2SO_4 solution there was no detectable loss. For example, in 2.5 N HCl after 90 months at 38° only 60 per cent of the cystine remained, whereas in 2.5 N H_2SO_4 under the same conditions, 100 per cent of the original cystine was still present. In both cases the fraction of the original optical activity remaining was about the same; *i.e.*, simple racemization had proceeded to about an equal degree in each.

The solutions all gave negative nitroprusside tests, negative tests for ammonia and Van Slyke amino nitrogen values (3 minute reaction) corresponding to the original amounts of cystine weighed in, regardless of loss as shown by the Folin-Marenzi determination. In all HCl solutions amounts of inorganic sulfate were present, varying from 3 to 36 per cent of the original cystine present, but these were, in all cases, much less than the losses of cystine. Determinations of total sulfur showed that no loss as H_2S had taken place.

Cysteic acid has been isolated as the principal product of the reaction, but other compounds with free amino nitrogen intact must obviously also be present. The identity of the cysteic acid was established by micro analysis, "melting point," optical activity, and by titration of the sulfonic acid group.

The reaction appears to be one of atmospheric oxidation, probably by intermediate formation of halide compounds. The usual traces of iron and copper which these solutions contained may possibly act as the catalyst in the oxidation.

THE THERMAL DECOMPOSITION OF METHIONINE IN ACID SOLUTION

By LEWIS W. BUTZ

(From the Laboratory of Physiological Chemistry, University of Illinois, Urbana)

Since we have found that the Folin-Marenzi and Sullivan methods give different values for the cystine content of insulin, we have been interested in studying these methods in some detail. Sullivan[5] has already reported that a mixture of amino acids such as exists in casein without cystine or other sulfur compounds gives, on heating with hydrochloric acid, a mixture which reacts strongly positively in the Folin-Marenzi test for cystine. We have heated similar and other mixtures of amino acids with sulfuric acid, and have obtained thereby products chromogenic with respect to the Folin-Marenzi reagent. From experiments with the individual amino acids we have concluded that tryptophane is the precursor of the chromogenic products in such mixtures.

In the study of the behavior of individual amino acids in the Folin-Marenzi method, we have encountered a very interesting decomposition of methionine, which leads to the formation of products chromogenic with the reagent of this method. The optimum conditions for the formation of these chromogenic products are rather more vigorous than those usually employed in protein hydrolysis. As yet the possible significance of the finding with respect to protein analysis has not been worked out. We intend to study this in detail later, but for the time being we are concerned with the decomposition observed. The latter apparently cannot be brought about by heating with hydrochloric acid.

By heating methionine with fairly strong sulfuric acid there is formed at least one non-volatile disulfide, together with traces of dimethyl disulfide. Upon neutralization and concentration of the sulfuric acid solution, a colorless compound was isolated, crystallizing from water in beautiful hexagonal plates and decomposing at 255–260° without melting. From the properties of the substance and the analytical figures for C, H, N, and S, the conclusion was drawn that the compound is the next higher homologue of cystine, $C_8H_{16}O_4N_2S_2$. The name homocystine is suggested for it. It is

[5] Sullivan, M. X., *Pub. Health Rep., U.S.P.H.S.*, suppl. 89 (1931).

possible that both a racemic and a meso form are produced in the decomposition of the racemic methionine. We hope to separate these if present. A study of the mechanism of the reaction of formation and a more rigid proof of structure by synthetic means will be made. The compound isolated does not react in the Sullivan method for cystine, thus furnishing another example of the amazing specificity of the Sullivan test.

OXIDATION OF DISULFIDES

By KAMENOSUKE SHINOHARA

(From the Research Institute of the Lankenau Hospital, Philadelphia)

Organic sulfur compounds play such an important rôle in physiological processes that they have recently attracted the attention of a number of workers. Clear understanding of the rôle of sulfur compounds, however, greatly depends upon a knowledge of their oxidation processes. The oxidation of thiol compounds has been fairly well studied, except for the intermediate compound in oxidation-reduction equilibrium with them, concerning which there is some divergence of opinion; but the knowledge of the oxidation of disulfides, which is just as important physiologically if not more so, is so far limited to qualitative work The reason, on the one hand, is the lack of a proper means of attack of this problem, and, on the other, the complexity of the processes.

Recently the author[6] has reported that disulfides are slowly oxidized by iodine to their respective sulfonic acids and then to sulfuric acid still more slowly. The slow rate of oxidation by iodine and the sensitivity of iodine titration offer a practical method for the study.

The author, therefore, started to study the kinetics of the reaction by this means.

As will be seen in the previous report, oxidation of disulfides by iodine in aqueous solution is in accordance with the following stoichiometrical equation:

$$RSSR + 5I_2 + 6H_2O = 2RSO_3H + 10HI \tag{1}$$

The reaction, however, does not proceed as a hexamolecular reaction in reality. This fact can be clearly understood from the kinetic standpoint.

[6] Shinohara, K., *J. Biol. Chem.*, **96**, 285 (1932).

The possibility is, then, a consecutive bimolecular reaction. This reaction in its simplest form is expressed by the following equations:

$$\text{RSSR} + \text{I}_2 + \text{H}_2\text{O} = 2\text{RSOH} + 2\text{HI} \tag{2}$$
$$\text{RSOH} + \text{I}_2 + \text{H}_2\text{O} = \text{RSO}_2\text{H} + 2\text{HI} \tag{3}$$
$$\text{RSO}_2\text{H} + \text{I}_2 + \text{H}_2\text{O} = \text{RSO}_3\text{H} + 2\text{HI} \tag{4}$$
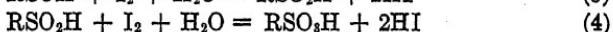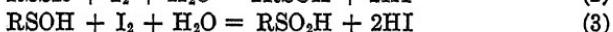
Each step may be preceded by other intermediate steps and especially in case of Equation 2 there is some support for assuming the following reaction as an intermediate one:

$$\text{RSSR} + \text{H}_2\text{O} = \text{RSOH}_2\text{SR} \tag{5}$$

The results of the kinetic study, however, can be represented by the above three reactions and their kinetic equations which are respectively:

$$\frac{dx}{dt} = k_1(A - x)\,(B - x - 2y - 2z) \tag{a}$$

$$\frac{dy}{dt} = k_2(x - y)\,(B - x - 2y - 2z) \tag{b}$$

$$\frac{dz}{dt} = k_3(y - z)\,(B - x - 2y - 2z) \tag{c}$$

where

A is number of mols of RSSR per liter initially present
B " " " " " iodine " " " "
x " " " " " RSSR which disappeared in t time
y " " " " " RSOH " " " " "
z " " " " " RSO$_2$H " " " " "
k_1, k_2, k_3 are velocity constants of the respective reactions

Without knowing the values of k_1, k_2, and k_3 the equations cannot be integrated. However, at the early stage of the reaction, when y and z are approximately 0, the reactions of Equations 3 and 4 disappear and the kinetic equation is reduced to:

$$\frac{dx}{dt} = k_1(A - x)\,(B - x) \tag{d}$$

The equation gives, on integration

$$k_1 = \frac{2.303}{t(A - B)} \log \frac{B(A - x)}{A(B - x)} \tag{e}$$

A series of experiments has been carried out and the results have confirmed this proposition. The value of k_1 has been found to be approximately 1.15 under the experimental conditions.

THE ISOLATION AND CHARACTERIZATION OF MESOCYSTINE

BY HUBERT S. LORING AND VINCENT DU VIGNEAUD

(From the Laboratory of Physiological Chemistry, University of Illinois, Urbana)

The question as to the identity of inactive cystine obtained by racemization of the naturally occurring *l*-cystine with boiling 20 per cent hydrochloric acid has been the subject of many investigations. Whether this material was the meso or racemic form or a mixture of the two has been the point of contention. That the racemic cystine was actually present was conclusively shown by du Vigneaud and Hollander,[7] who isolated pure *d*-cystine by subjecting the inactive cystine to resolution. This work, however, could not show whether or not the meso form was present as well. The inactive cystine was, therefore, further studied in an attempt actually to isolate the mesocystine in pure form.

Recently, we have reported[8] the isolation of two inactive forms of cystine and tentatively assumed the more soluble one to be the meso isomer. The less soluble form was identical with racemic cystine obtained by mixing equal parts of pure *d*- and *l*-cystine.

On the basis of further work, we can now conclude that the more soluble form is actually the internally compensated or meso enantiomorph. This conclusion was based on the failure of further attempts to resolve the more soluble form, whereas under the same conditions clean cut resolution of the racemic form was obtained. The strychnine salt of the formylcystine was found to be

[7] du Vigneaud, V., and Hollander, L., *Proc. Soc. Exp. Biol. and Med.*, **28**, 46 (1930).

[8] Loring, H. S., and du Vigneaud, V., *Proc. Soc. Exp. Biol. and Med.*, **29**, 41 (1931).

more satisfactory than the brucine salt which we had previously used.

We have furthermore characterized these isomers of cystine by the preparation of a number of derivatives. The solubility of all four isomers of cystine has also been determined. Some evidence for the interconversion of the meso and racemic cystine has been investigated.

CYSTINE STUDIES IN ARTHRITIS

By M. X. SULLIVAN and W. C. HESS

(From the Chemo-Medical Research Institute, Georgetown University, Washington)

The cystine content of the finger nails of normal individuals was found to vary from 11 to 13 per cent. In hypertrophic arthritis the cystine content of the nails was normal; in atrophic and infectious arthritis the cystine content runs low.

One case of infectious arthritis was found to have as low as 7 per cent cystine. Followed month by month for 6 months with improvement in general condition the cystine content of the finger nails rose from 7 per cent to over 11 per cent when the signs and symptoms of arthritis had disappeared.

SULFUR DISTRIBUTION IN PROTEINS

By HARRY D. BAERNSTEIN

(From the Laboratory of Physiological Chemistry, University of Wisconsin, Madison)

Three fractions of sulfur are determined; namely, sulfhydryl, disulfide, and methionine sulfur. The first fraction represents the reducing power of a protein digest toward iodine in the presence of 2 N HCl. The second is determined by treating the digest with zinc in order to reduce disulfide groups to sulfhydryl and then estimating the total sulfhydryl. The difference between this fraction and the first is called disulfide fraction. Methionine is determined by boiling the protein with hydriodic acid and absorbing the methyl iodide in excess of alcoholic silver nitrate. Titration of the silver remaining unprecipitated gives the data for calculating methionine sulfur.

The methods have been standardized on pure amino acids and mixtures containing gelatin in addition.

The average recovery of the sulfur of thirty-two purified proteins was 100.1 ± 7.1 per cent.

The range of sulfhydryl sulfur was 0.0 to 36.0 per cent of the total sulfur, disulfide from 4.6 to 69.0 per cent, and the methionine sulfur from 26.0 to 77.0 per cent.

Casein contains 12.8 per cent of its sulfur as sulfhydryl, 8.6 per cent as disulfide, and 77 per cent as methionine. The best figures from the literature account for only 20 per cent of the sulfur of casein.

Egg albumin contains 18.8 per cent of its sulfur as sulfhydryl, 19.0 per cent as disulfide, and 61.4 per cent as methionine. In the literature only 26.7 per cent of the sulfur of this protein is accounted for.

ANALYSIS OF EGG-SHELL KERATIN

By HERBERT O. CALVERY

(*From the Laboratory of Physiological Chemistry, Medical School, University of Michigan, Ann Arbor*)

The keratin was prepared from the shells of fresh eggs from white Leghorn hens. The white and yolk were removed and the shells washed with water, extracted with 3 to 5 per cent hydrochloric acid to remove the mineral matter, and finally digested with pepsin in hydrochloric acid solution. The membranous material (keratin) remaining after this treatment was washed first with water, then with alcohol, and finally with ether. It was then dried in a vacuum desiccator over sulfuric acid and weighed. 156 gm. of keratin were obtained from approximately 800 eggs.

The keratin was analyzed for moisture, ash, sulfur, nitrogen, tyrosine, tryptophane, cystine, arginine, histidine, lysine, glutamic acid, and aspartic acid.

A NEW METHOD FOR THE DETERMINATION OF ALBUMIN AND GLOBULIN IN BLOOD SERUM

By JOSEPH M. LOONEY

(*From the Memorial Foundation for Neuro-Endocrine Research and the Worcester State Hospital Laboratories, Worcester, Massachusetts*)

The method of estimating serum globulin and serum albumin is based on the determination of the tyrosine and cystine content

of the respective proteins. The tyrosine is determined by the method of Folin and Marenzi, and the cystine by the method of Folin and Looney. The following values are taken as the basis of calculation: serum albumin, tyrosine 4.67 per cent, cystine 6.06 per cent; serum globulin, tyrosine 6.70 per cent, cystine 0.70 per cent.

For the analysis, 2 ml. of serum are added to 10 ml. of metaphosphoric acid, in a 15 ml. graduated Pyrex test-tube. The tube is stoppered, shaken vigorously, and then centrifuged for 10 minutes. The supernatant liquid is poured off, the proteins are washed with 10 ml. of distilled water, and again are centrifuged. 2.5 ml. of a 14 N solution of sulfuric acid are added, the tube is closed with a piece of heavy tin-foil, and is placed in an autoclave at 130° for 3 hours. After cooling, the volume is adjusted to exactly 10 ml. and one-half of the digestion fluid is taken for each analysis.

Calculation—Let A equal the amount of cystine in mg. and B the amount of tyrosine in mg. Per cent albumin equals $1.79A - 0.187B$; per cent globulin, $1.62B - 1.25A$.

THE CLINICAL PARTITION OF BLOOD PROTEIN BY SCOPOMETRY. II. METHOD

By WILLIAM G. EXTON and ANTON R. ROSE

WITH THE ASSISTANCE OF FRED SCHATTNER, FRANCES EDEL, AND MARY McCARTHY

(From the Laboratory and Longevity Service of The Prudential Insurance Company of America, Newark)

Our experience with a protein precipitate exhibiting consistent relations between protein concentration and light transmission as measured by the photoelectric scopometer led us to develop the following method of separating the protein components of blood.

Total protein is determined on 100 times diluted plasma by precipitating with equal volumes of sulfosalicylic acid reagent and measuring with the photoelectric scopometer.

For the protein fractionation, make up a neutralized saturated ammonium sulfate solution by adding 5 cc. of N sodium hydroxide to each liter. This is then diluted in such a way that 9 cc. added to similar tubes holding 1 cc. of 5 times freshly diluted plasma and 3 standard drops of acetone make the final concentration in the

six successive tubes 7:20, 8:20, 10:20, 12:20, 14:20, and 17:20 saturation. After standing at least 1 hour, the contents of the tubes are filtered through 7 mm. Munktell 00 paper, drained completely, and washed by allowing 4 cc. of ammonium sulfate solution of like concentration to flow around the inner edge of the filter paper. After washing, 20 cc. of saline solution are poured through the filter in clean, dry test-tubes to dissolve the protein and allowed to pass through a second time to insure homogeneity.

TABLE I

Protein Partition. Normal Plasma

	Total protein	Globulin	Fibrinogen	Albumin/Globulin
	mg. per 100 cc.	*per cent*	*per cent*	
Maximum............	8460	42.0	5.4	2.8
Minimum	6600	26.5	2.0	1.4
Quartiles............	8200		3.3	2.6
	7100		3.1	2.0
Average............	7730	31.1	3.4	2.3

	Globulin No.				Albumin No.		
	I	II	III	IV	I	II	III
Maximum............	4.6	9.8	14.7	15.0	32.8	38.9	27.3
Minimum............	0.0	3.7	10.0	3.2	17.2	21.8	4.2
Quartiles............	2.7	8.2	13.1	8.2	27.5	34.8	18.5
	1.7	4.3	11.6	5.9	21.6	22.9	9.1
Average............	2.0	6.5	12.3	7.3	24.8	29.6	14.2

The six protein precipitates now represent a 100 times dilution and are determined in the same way as the total protein.

It is patent that the division between albumin and globulin is clearest cut at 12:20 saturation. When the protein in preceding ones is subtracted from the value of each tube we call the first four Globulins I, II, III, and IV and the last three Albumins I, II, and III. Globulin I is the 7:20 tube after the fibrinogen is subtracted. The difference between the accumulated protein in the 17:20 tube and the total protein is recorded as Albumin III.

Fibrinogen has been determined separately by salting out with half saturated sodium chloride, but for clinical purposes a 6:20 tube represents the fibrinogen fraction free from other protein, and this offers a simpler and quicker alternative method. We are experimenting with direct precipitation in the hope of avoiding the necessity of filtering and washing.

As precipitations with sulfosalicylic acid may be affected by the presence of salts, the concentration of ammonium sulfate should never exceed 1:40 and sodium chloride 1:6 saturation.

Table I gives results from the plasma of twenty adult normals. Nearly a hundred pathological bloods have also been partitioned with results very different from those of the normals. Some of these are now indicated but will be discussed in later papers.

PRECIPITATION OF SOY BEAN PROTEINS AT VARIOUS CON-CENTRATIONS OF AMMONIUM SULFATE

By D. BREESE JONES and FRANK A. CSONKA

(*From the Protein and Nutrition Division, Bureau of Chemistry and Soils, United States Department of Agriculture, Washington*)

Glycinin, the chief protein of the soy bean, was first prepared by Osborne and Campbell by saturating a 10 per cent sodium chloride extract of the meal with ammonium sulfate, redissolving the resulting precipitate, and dialyzing the solution.

The precipitation limits of glycinin with ammonium sulfate are not given in the literature. Knowledge of this property would be helpful in obtaining purer preparations of glycinin.

By fractional precipitations at definite concentrations of ammonium sulfate within a range of 33 to 70 per cent of saturation, five protein fractions have been obtained. In order to determine which fraction represents glycinin, a salt extract of soy bean meal was dialyzed. From the protein fraction which precipitated, two globulins were separated. One is precipitable from a 10 per cent sodium chloride solution by ammonium sulfate at 55 per cent of saturation and does not coagulate even at boiling temperature. These properties agree with those given by Osborne for glycinin. It is of interest to note that the isoelectric point of this globulin was found to be at pH 5.2, which is considerably higher than that found for unfractionated glycinin. The higher value now found

brings this protein within the isoelectric point range found to be characteristic for globulins, as given in a previous publication from this laboratory.

The other globulin precipitates at 65 per cent ammonium sulfate saturation, and coagulates at 72°.

The dialysate contained three proteins precipitable by ammonium sulfate at 38, 49, and 69 per cent of saturation. Two of these proteins have the unusual properties of having both a high degree of solubility and a relatively low degree of precipitability by ammonium sulfate.

CHEMISTRY OF THE INTEGUMENT

IV. COLLAGEN

By WITHROW MORSE

(From the Röhm and Haas Company, Inc., Philadelphia)

Collagen was obtained from corium deprived of epidermis and flesh. In order to purify the corium from other proteins, etc., it was permitted to autolyze for 60 days, then subjected to tryptic substances. All extractable substances giving positive biuret, ninhydrin, sulfur, xanthoproteic, and tryptophane reactions were removed. When boiled with aqueous solution at pH 4.6, gelation occurred after cooling. It was observed that gelatin arose only after prolonged boiling. With the exception of the biuret test, all qualitative reactions mentioned above were negative and the substance was practically refractory to tryptic enzymes. Collagen was not dispersed by means of urea nor by inorganic thiocyanates. Tyrosine is definitely present.

THE EFFECT ON THE TOXICITY OF EGG WHITE OF VARIOUS HEAT TREATMENTS

By HELEN T. PARSONS

(From the Department of Home Economics, University of Wisconsin, Madison)

Various types of fresh and dried egg white were subjected to gradated temperatures and duration of heating and then dried before incorporating into the diet of rats in which pronounced

pellagra-like manifestations had been produced on a diet high in raw egg white.

Samples of egg white from fresh eggs and from cold storage eggs were about equally detoxified by the same degrees of heat treatment. Chinese dried egg white, reconstituted by the addition of water in the proportions present in fresh egg white, required about 18 times the length of heating at 80° that the other two types required for detoxification. However, egg white dried in the laboratory at room temperature before a fan and reconstituted required no more time for detoxifying than fresh egg white. If, during the reconstituting of Chinese egg white it was soaked (at 3°) for a week instead of from 2 to 4 hours, the time of heating required for detoxification was materially shortened. All types of egg white insufficiently heated were more toxic when fed at a 66 per cent than at a 40 per cent level. Fresh egg white coagulated by alcohol, leached in running water for 50 hours, and fed raw was as toxic as before treatment.

THE RELATION OF CERTAIN 5-CARBON AMINO ACIDS TO GROWTH

By RUTH REDER St. JULIAN AND WILLIAM C. ROSE

(From the Laboratory of Physiological Chemistry, University of Illinois, Urbana)

Investigations have been conducted in order to determine the dietary importance of (1) the dibasic amino acids, and (2) the prolines. The results show that after the removal from hydrolyzed casein of aspartic, glutamic, and hydroxyglutamic acids, the remaining mixture of amino acids, when fed at a 9 per cent level, induces moderately good growth which is not improved by the addition of the missing components.

Furthermore, proline and at least the major part of the hydroxyproline have been removed from three hydrolyzed proteins, namely casein, lactalbumin, and edestin, by forty extractions with absolute ethyl alcohol. Each of the resulting materials, when administered at a 9 per cent level, in diets adequate in other respects, permitted just as satisfactory growth as was obtained in control experiments by* the inclusion of proline in the basal ration.

In view of the theoretical possibility of the prolines, the glutamic acids, and the ornithine part of arginine playing a mutually interchangeable rôle in nutrition, all five amino acids were removed as completely as possible from hydrolyzed casein.   The residue was administered at a 11.5 per cent level.   Growth at a rate of approximately 1.0 gm. per day was secured.   This was not improved by the addition of glutamic acid, proline, arginine, and aspartic acid (which was precipitated along with the glutamic acids).

The above results appear to indicate that the amino acids in question are neither indispensable components of the diet, nor interchangeable in metabolism.

EFFECT OF TRYPTOPHANE ON GAIN IN WEIGHT WHEN USED TO SUPPLEMENT CORN-MEAL PROTEIN

By C. A. CARY and C. F. HUFNAGEL

(From the Research Laboratories, Bureau of Dairy Industry of the United States Department of Agriculture, Beltsville, Maryland)

Recently Mitchell and Smuts[9] published work from which they came to the conclusion that tryptophane, when used along with corn-meal as the only source of protein in the rations of growing rats, does not promote an increase in the rate of gain in body weight but actually depresses this rate.   The high degree of probability with which this depressant effect of tryptophane is demonstrated leaves little doubt that it is associated with the tryptophane that they used, although this constituted only 0.25 per cent of the ration, and the food consumptions of their rats on this ration and of comparable controls were practically the same. Their observation raises the question as to whether this depressant effect is characteristic of tryptophane or not; and, if it is not characteristic of tryptophane, the question naturally arises as to whether this amino acid would then similarly fail to supplement corn-meal proteins.

We have carried out three experiments along this line involving sixteen pairs of rats (litter mates comparable as to sex, initial weight, and feed consumed).   In none of these experiments does the mean difference in gain in weight between comparable rats receiving tryptophane and controls not receiving it exceed the

[9] Mitchell, H. H., and Smuts, D. B., *J. Biol. Chem.*, **95**, 263 (1932).

probable error of this result. Not all of our experiments are equally free from factors which complicate their interpretation; but they leave little doubt of the correctness of this conclusion.

THE OCCURRENCE OF FATTY LIVERS IN RATS FED DIETS CONTAINING LIVER

By N. R. BLATHERWICK, E. M. MEDLAR, PHOEBE J. BRADSHAW, ANNA L. POST, AND SUSAN D. SAWYER

(*From the Biochemical Laboratory and the Hegeman Memorial Laboratory of the Metropolitan Life Insurance Company, New York*)

We have recently reported that rats fed diets containing dried, whole liver develop fatty livers. These livers contain large amounts of cholesterol esters and fatty acids but do not have an increased content of lecithin. These experiments were interpreted as indicating the presence of a substance in dried, whole liver, the ingestion of which causes the development of fatty livers. It was shown that feeding residues from aqueous and from alcoholic extraction of liver does not produce fatty livers.

It has since been found that this fatty condition is produced when raw liver is fed. Other experiments show that a liberal allowance of carbohydrate in the diet does not prevent the occurrence of fatty livers. Additional experiments reveal that the development of this condition is well marked within a period of 1 week. Feeding a dried 70 per cent alcoholic precipitate of an aqueous extract of liver does not cause this deposition of fat. Some chemical properties of the various fat fractions have been determined.

FATTY ACIDS OF LIVER LECITHIN

By RUTH H. SNIDER AND W. R. BLOOR

(*From the Department of Biochemistry and Pharmacology, The University of Rochester School of Medicine and Dentistry, Rochester, New York*)

A study was made of the nature of the fatty acid mixture found in liver lecithin by the use of relatively simple methods of separation and identification. The methods used were (*a*) the Twitchell lead salt-alcohol method of separation of solid and liquid acids; (*b*) the separation of the unsaturated acids by means of their bromine

addition products; (c) the method of Cocks, Christian, and Harding for separation of the solid unsaturated acids from the solid saturated acids.   All these procedures were modified somewhat from the published technique.

The acids found with their approximate percentages of the total fatty acids were as follows:

Total fatty acids
per cent

Solid saturated...36.0
   M.p. 63°; mol. wt. 275; I.N. 4
Solid unsaturated... 5.0
   M.p. 43; I.N. 95
Liquid unsaturated..54.68
   I.N. Found, 215; calculated from bromine content, 201
   4-bond acid (arachidonic) 15.34
   3- "   "
   2- "   "   25.47
   1- "   "   13.87

95.68

RELATIONSHIP BETWEEN DEGREE OF UNSATURATION AND COMPOSITION OF LIPIDS IN ANIMAL TISSUES

By ROBERT GORDON SINCLAIR

(From the Department of Biochemistry and Pharmacology, The University of Rochester School of Medicine and Dentistry, Rochester, New York)

In papers recently published in *The Journal of Biological Chemistry* the author has shown that both the amount and type of fat in the diet exert a very pronounced and characteristic influence on the degree of unsaturation of both the phospholipids and neutral fat in the tissues of the rat.   For the proper understanding of the bearing of these observations on the still unsettled question of the function of the tissue phospholipids, it was found to be essential to have definite information as to the relative proportion of saturated and unsaturated fatty acids and of the various unsaturated acids in phospholipids of widely different degrees of unsaturation.

To supply that need, the constituent fatty acids of the total phospholipids from the musculature and visceral organs of rats which had been fed on various distinctive diets were separated into saturated and unsaturated fractions by the lead salt-alcohol method.   The unsaturated acids were brominated and the bro-

mides separated into petroleum ether-soluble, ether-soluble, and ether-insoluble fractions. In some cases, a similar separation was carried out on the fatty acids present in the tissues as neutral fat.

The data thus far obtained show that the ratio of the saturated to unsaturated fatty acids in phospholipids remains constant at about 31:69 irrespective of the mean degree of unsaturation, and that, in consequence of this constancy in the percentage of saturated acids, the mean degree of unsaturation is governed entirely by the relative proportion of the various unsaturated acids. An increase in unsaturation is accompanied by a decrease in those fatty acids which yield bromides soluble in petroleum ether, and by an increase in those fatty acids which yield both ether-soluble and ether-insoluble bromides. The characteristic influence of the diet fat on the composition of the tissue phospholipids appears to be due, not to a direct incorporation of ingested fatty acids into the phospholipid molecule, but rather to some indirect mechanism, the nature of which is still obscure.

The observations on the fatty acid distribution in the neutral fat are in sharp contrast to those in the phospholipids. In neutral fat an increase in degree of unsaturation is accompanied by a decrease in the percentage of saturated acids and an increase in the percentage of oleic and linoleic acids. Even when the diet contains about 20 per cent of cod liver oil, which is rich in the more highly unsaturated fatty acids, the percentage of such highly unsaturated acids in neutral fat is quite small.

THE HIGHLY UNSATURATED FATTY ACIDS IN BUTTER

By H. C. ECKSTEIN

(*From the Laboratory of Physiological Chemistry, Medical School, University of Michigan, Ann Arbor*)

The presence of fatty acids more highly unsaturated than oleic acid has been demonstrated in butter fat. By use of the bromination method crystalline bromides having the characteristics of tetra-, hexa-, and octabromides were isolated. From the weights of ether-soluble, petroleum ether-insoluble bromides, it was calculated that the butters analyzed contained from 0.17 per cent to 0.25 per cent of linoleic acid. The ether-insoluble bromides proved

to be a mixture of hexa- and octabromides, as evidenced from their solubilities. The major fraction, however, appears to be made up of hexabromides. For that reason they are tentatively considered as bromides of linolenic acid. On this basis the amounts of this acid in the fat were found to range from 0.09 to 0.17 per cent. When linoleic acid in the form of the methyl ester was added to butter fat, only 40 per cent of the theoretical amount of crystalline bromides could be isolated, and when the ester of linolenic acid was added, the weights of bromides were only 16 per cent of the theoretical in one case and 19 per cent in another. When the esters were saponified and brominated in the absence of other fatty substances, 43 per cent of the theoretical amount of tetrabromides was secured and only 21 per cent of the expected amount of hexabromides.

These facts show that the method employed gives low results, and it is obvious, therefore, that butter contains larger amounts of the more highly unsaturated acids than those reported here.

A STUDY OF THE GAS EXCHANGE OF RATS SUFFERING FROM A DEFICIENCY OF UNSATURATED FATTY ACIDS

By GEORGE O. BURR AND A. J. BEBER

(From the Department of Botany, University of Minnesota, Minneapolis)

A continuous sampling device has been constructed which collects and holds samples of dry gas for analysis in the Carpenter-Haldane apparatus. The metabolic rates and respiration quotients of rats have been followed 24 to 48 hours, each sample being collected every 4 hours. The collections have been made under normal conditions with food in the chamber as well as during fasting.

The findings of Wesson and Burr[10] are confirmed that these rats have exceptionally high respiratory quotients and metabolic rates. These newer results show that under natural food conditions (no fasting) the respiratory quotients of fat-starved rats remain above 1.00 (averaging 1.05) for 12 to 16 hours each day. Then when they stop eating, the quotient drops extremely rapidly to one representing the burning of much fat. Rats which have been cured with 10 drops daily of any effective oil show a daily

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[10] Wesson, L. G., and Burr, G. O., J. Biol. Chem., 91, 525 (1931).

course of metabolism approximating that of stock animals on stock diet; *i.e.*, their respiratory quotients rarely reach 1.00 and the fall of quotient with fasting is much slower.

As a point of general interest in metabolism studies, it is shown that metabolic rates fall to a minimum without any fasting, while the rat is still absorbing food from the intestinal tract and the quotient is above 0.85.

THE NUTRITIVE VALUE OF PURE FATTY ACID ESTERS

By WARREN M. COX, Jr.

(*From the Department of Pediatrics, the Johns Hopkins Medical School, Baltimore*)

The author undertook to study the nutritive properties of the individual components of a natural fat. Coconut oil was taken as a starting point. First, the whole oil and then various fractions obtained from it were incorporated in the diets of growing rats. A complete diet was used in which 77 per cent of the calories was supplied as fat; only the fat component of the diet was varied.

It was found that the ethyl esters of coconut oil gave quite as satisfactory growth as the triglycerides. The ethyl esters were first separated into three fractions by distillation, and these used in the feeding experiments. The esters boiling below ethyl laurate, although they enabled the animals to survive and maintain their weight, did not permit normal growth. When ethyl laurate was fed, the animals almost invariably died within 10 days. The higher boiling esters permitted normal growth. The substitution of a small quantity of higher boiling esters for a fraction of the ethyl laurate was sufficient to prevent death and to permit growth. The fatal outcome does not appear to be due to the lack of some essential unsaturated fat constituent.

The esters of the individual acids of these fractions were prepared and supplemented with synthetic esters so as to complete the saturated series from acetic acid to stearic acid. Over a 30 day period only two esters allowed fairly normal growth: glyceryl acetate and ethyl myristate; one, ethyl caprylate, allowed survival of the animals but inferior growth; ethyl butyrate, ethyl laurate, ethyl palmitate, and ethyl stearate resulted in the death of all animals within 10 days; three of the four animals on ethyl

caproate, and two of the four on ethyl caprate, died; the survivors exhibited mediocre growth.

SOME FURTHER STUDIES OF THE FACTORS LEADING TO THE ABNORMAL IODINE VALUES OF CHOLESTEROL

By J. O. RALLS

(From the Department of Biological Chemistry, University of Buffalo Medical School, Buffalo)

Studies were made of the halogen consumed, of the halogen acid produced during the reaction, and of the halogen actually combined with the organic substance when commercial cholesterol, pure cholesterol, cholesteryl acetate, cholesteryl benzoate, triphenylmethylcholesteryl ether, and their dibromo derivatives were allowed to react with the Hanus reagent, CCl_4 instead of $CHCl_3$ being used as the solvent. The loss of halogen acid from the dibromo derivatives was followed in the presence of glacial acetic acid with and without IBr. The results were plotted against the time of reaction.

The results showed that there was a continuous consumption of halogen in all cases; that the organically bound halogen in the non-brominated substances reached a limiting value of 80 per cent of the theoretical or an average of 1.6 equivalents per mol of cholesterol or its derivatives; that in the case of dibromo derivatives in the presence of IBr in glacial acetic acid, halogen acid was lost causing the bound halogen acid to tend toward the figure of 1.6 equivalents per mol. In the absence of IBr the same general results were obtained except that the rate of loss was less.

When the secondary alcohol group was free, a ketone was isolated as a 2, 4-dinitrophenylhydrazone which contained no halogen and which had the same melting point, mixed and unmixed, and on analysis gave the same molecular weight as the 2,4-dinitrophenylhydrazone of cholestenone.

THE UNSAPONIFIABLE LIPIDS OF BEEF LIVER

By F. C. FREYTAG and H. GREGG SMITH

(From the Biochemical Laboratory, State University of Iowa, Iowa City)

The unsaponifiable constituents of beef liver lipids were fractionated by means of their differential solubility in various organic solvents. Physical properties (melting point and specific rota-

tion) of the various sterol fractions and their derivatives (acetate, benzoate, bromide) seem to indicate that one cholesterol is present and that it is identical with cholesterol obtained from other sources. Small amounts of nitrogen-containing substances were obtained and these are in process of identification. Only a small amount of ergosterol was found and this was partially concentrated in the later and more soluble fractions.

Three non-sterol fractions were obtained. Two of these contain considerable quantities of vitamin A, as shown by the SbCl₃ color test and measurements of ultra-violet absorption by sector photometer. By vacuum distillation of these fractions the vitamin was concentrated in the lower boiling fractions (150–170° at 0.5 mm.), but with considerable destruction. On the basis of the color test and ultra-violet absorption, beef liver fat contains 3 to 5 times as much vitamin A as a commercial assayed and protected cod liver oil. Antioxygenic material was found in all of the non-sterol fractions, but concentration by vacuum distillation has thus far been only partially successful.

CEREVISTEROL, A STEROL ACCOMPANYING ERGOSTEROL IN YEAST

By EDNA M. HONEYWELL and CHARLES E. BILLS

(From the Research Laboratory, Mead Johnson and Company, Evansville, Indiana)

Cerevisterol, $C_{29}H_{49}O_3$, is a stable, hexane-insoluble sterol obtained from the acetone mother liquors after the extraction of ergosterol from yeast. It melts at 265.3°, has $[\alpha]_{5461}^{25} = -57.4°$ in chloroform, and shows feeble absorption at 248 millimicrons. It develops no antiricketic potency upon irradiation. It forms a diacetate, m.p. 170.5°, $[\alpha]_{5461}^{25} = -162.9°$. It has two double bonds, one of which appears to be the $\Delta^{1,2}$ linkage of ergosterol.

EFFECT OF STEROL CONTENT OF THE DIET UPON CYCLIC VARIATIONS IN BLOOD CHOLESTEROL IN WOMEN

By RUTH OKEY and DOROTHY STEWART

(From the Laboratory of Household Science, University of California, Berkeley)

The four women students who were subjects for this study were placed for periods of 4 to 5 weeks at a time on each of three ade-

quate diets which were constant from day to day.   The amounts
and varieties of bread, milk, cream, butter, mayonnaise, meat, etc.,
were the same for all three diets.   But Diet I (low cholesterol)
contained only about 0.75 gm. of cholesterol per day.   Diet II
contained added egg yolk and liver with a total cholesterol con-
tent of about 3.1 gm. per day, while Diet III contained sufficient
added cholesterol as such to make a total of about 3.2 gm. per day.

The trends of the curves for blood cholesterol as obtained from
before breakfast samples, taken three times weekly were altered
only slightly by sterol intake and were, as previously observed,
high just preceding menstruation, with a fall near the time of on-
set and a rise afterwards.

Mean values for the month were consistently, if slightly, higher
with the high food cholesterol intake (168 ± 3.3 mg., 41.8 per cent
esterified) than on the low cholesterol diet (150 ± 2.9 mg., 38 per
cent esterified), while the diet with added cholesterol gave a some-
what less pronounced increase in blood cholesterol level (160
± 2.4 mg., 41.5 per cent esterified).

FACTORS LIMITING THE CAPACITY OF A DOG FOR WORK

By J. H. TALBOTT, L. J. HENDERSON, H. T. EDWARDS,
AND D. B. DILL

(From the Fatigue Laboratory, Morgan Hall, Harvard University, Boston)

A study has been made of the performance of a dog trained to
run on a treadmill with a pitch of 10°, motor-driven at rates of 125,
156, 187, 233, and 311 meters per minute.   This dog can maintain
the fastest pace 7 minutes, an athlete the slowest pace 6 minutes,
with approximately equal lactic acid accumulation in each case.
Thus a dog can transport, per unit of body weight, 2½ times more
oxygen than an athlete, if the efficiency is the same.

At the first two rates, provided external temperature is low,
blood lactic acid remains at the resting level and exhaustion is
accompanied by hypoglycemia.   With adequate intake of fuel
(glucose candy by mouth) and of water during semihourly 5
minute rest periods, the dog ran 13 hours at the 125 meter rate and
6½ hours at the 156 meter rate.   The blood sugar level remained
normal or high in both cases.   Without fuel, exhaustion came in
6 hours and in 4¼ hours, respectively, with blood sugar concentra-

tions of 66 and 50 mg. per 100 cc., respectively. In the latter case 50 gm. of candy restored the blood sugar, revived the dog, and within 10 minutes it began running willingly, continued for 75 minutes, and was not exhausted when the experiment was discontinued.

At these speeds, but particularly at the next two higher speeds, unless the external temperature is low, the heat-dissipative mechanism fails. Rectal temperature and blood lactic acid increase together (reaching in one experiment 43.3° and 0.095 per cent, respectively) and the heart rate falls. Cardiotachometric records of the heart rate during exercise reveal a relation between energy output and rate which is linear, until a limiting value of about 300 beats per minute is reached. 10 seconds after work ends, the rate may have decreased one-third. This indicates that observations of the rate during recovery give unreliable information regarding its value during work.

THE COMPOSITION OF GASTRIC JUICE AS A FUNCTION OF ITS ACIDITY. SOME PROPERTIES OF THE PARIETAL SECRETION

By FRANKLIN HOLLANDER

(From the Department of Physiology, New York Homeopathic Medical College, New York)

It was found recently[11] that the maximum acidity of gastric juice which can be realized (i.e. in pure parietal secretion) corresponds to an isotonic solution of hydrochloric acid in which there is no fixed base. The present investigation was designed to confirm these characteristics of the parietal secretion, and to determine some of its other properties. Samples of gastric juice from several dogs with fundus pouch were mixed so as to yield five large composite samples of different acid concentrations. The total acidities of two of these, which had been made up from specimens containing only traces of mucus, were 155 and 158 mM; the other three acid values were 111, 136, and 144 mM. All five liquids were examined for a number of their chemical characteristics, with the following results.

As the total acidity increased from 111 to 158 mM, (1) the combined acidity decreased from 4 to 2 mM; (2) total chloride concen-

[11] Hollander, F., Proc. Soc. Exp. Biol. and Med., 29, 640 (1932).

tration rose from 139 to 163 mM, whereas the neutral chloride
values decreased from 28 to 5 mM; (3) total solids decreased from
0.35 to 0.12 per cent, organic solids from 0.15 to 0.04 per cent, and
ash (as chloride) from 0.20 to 0.08 per cent; (4) specific gravity
(25°) decreased from 1.002 to 1.001; (5) biuret and Hopkins-Cole
reactions were positive and of decreasing intensity in all but the
sample of highest acidity; in the latter they were so faint as to be
indecisive; (6) the Molisch reaction was negative throughout; (7)
determinations of phosphorus in the two samples of highest acidity
yielded negative results; (8) freezing point depressions of these
same samples were 0.614° and 0.628°; (9) approximate determina-
tions of their ammonia content indicated values around 0.5 mM.

From these results it may be concluded that the properties of the
sample of highest acidity approximate fairly closely those of the
parietal secretion.   Therefore, the parietal cells elaborate an iso-
tonic solution of hydrochloric acid which contains no neutral
chloride, phosphate, or any other inorganic material in significant
quantity.   Its content of organic matter, if at all significant, is
very small.   Its specific gravity is that of pure hydrochloric acid.
The formation from the lymph of such a fluid can be explained
more satisfactorily in terms of a membrane which is specifically
permeable to the hydrogen halides and water than by a Donnan
membrane effect or a series of chemical reactions, as has been
suggested by numerous investigators.

STUDIES ON THE TRANSPLANTED INTESTINAL LOOP

II. A HUMORAL INFLUENCE IN THE SECRETION OF INTESTINAL JUICE

By H. B. PIERCE, E. S. NASSET, AND JOHN R. MURLIN

(From the Department of Vital Economics, The University of Rochester,
Rochester, New York)

Sections of upper jejunum, 15 to 20 cm. in length, were trans-
planted under functionally hypertrophied mammary glands of
dogs.   The mesenteric blood vessels and nerves were left intact
and brought through an opening at the fore end of the incision.

The secretion of these loops was collected before and after food
and the enzyme content determined by following quantitatively
the digestion of sucrose, starch, peptone, and olive oil.

After a collateral blood supply had been established from the mammary vessels, the mesenteric nerves and vessels were cut, leaving the segment entirely isolated from the rest of the alimentary tract. After the second operation, a further study was made of the effect of food and other substances on the rate of secretion and on the enzyme content of the juice.

The effects of food were studied in two ways. (1) The animal was fed in the morning, the juice collected for 7 or 8 hours after feeding, and the results compared with other collections of similar length of time when the animal had not been fed. (2) The animal was fed at noon, a collection of the fasting juice being made in the morning and of the food juice in the afternoon.

The study of the secretion of these transplanted, isolated loops has shown that food causes an increase in the volume and enzyme content of intestinal juice, which appears to prove that the secretion is under humoral control. The conclusions are based upon the results of forty-six experiments with and without food, with three dogs, following the second operation.

GASTROINTESTINAL TEMPERATURE STUDIES

By JOSEPH S. HEPBURN, HARRY M. EBERHARD, ROWLAND RICKETTS, and CHARLES L. W. RIEGER

(From the Hahnemann Medical College and Hospital, Philadelphia)

A recording resistance thermometer was introduced into the stomach in the same manner as a stomach tube, into the upper intestine (duodenum and jejunum) as for a non-surgical biliary drainage, into the sigmoid through a signoidoscope. The temperatures obtained were the following. Stomach: 129 males, minimum 36.6°, maximum 38.8°, average 37.3°; 128 females, minimum 36.4°, maximum 39.0°, average 37.4°. Upper intestine: thirty-four males, minimum 36.7°, maximum 38.4°, average 37.3°; nineteen females, minimum 36.7°, maximum 37.7°, average 37.2°. Sigmoid: six cases, minimum 38.2°, maximum 38.6°, average 38.4°. In a group of subjects the gastric temperature was approximately 0.6° higher than the oral temperature, the intestinal temperature was always within 0.5 of the gastric temperature, and the sigmoidal temperature, on the average, 1.6° higher than the oral temperature. Ingestion of ice water (250 cc.) or ice cream (90 gm.) pro-

duced a decrease in gastric temperature, followed by a return to normal temperature. When ice water was given to 52 subjects, the average decrease was 14.1°, the average recovery time 38.9 minutes. Following ice cream in 51 subjects the average decrease was 3.7°, the average recovery time 32.6 minutes. Drinking a cup of hot coffee increased the gastric temperature as much as 9.4°. In 77 experiments, physiotherapeutic agents (electric pad, hot water bottle, infra-red lamp, diathermy, hot wet pack, ice bag) were applied over either the stomach or the upper intestines, usually for 1 hour; the changes in visceral temperature were within the limits of the normal changes in gastric temperature in a control series of forty-one experiments. Use of ice water in a test meal delayed the gastric emptying time by 15 to 30 minutes.

REVERSIBILITY OF PROTEIN DENATURATION IN ADSORPTION AND ELUTION

By MONA SPIEGEL-ADOLF

(From the D. J. McCarthy Foundation, Department of Colloid Chemistry, Temple University Medical School, Philadelphia)

For the better understanding of the denaturation of proteins and its reversibility, it seemed necessary to study conditions in which primary chemical changes of the proteins could be practically excluded. Adsorption of proteins by the means of surface-active substances seemed to meet this requirement to a certain extent. In conformity with prior authors, the proteins of the serum were adsorbed by aluminum hydroxide and mastic sol, but, for the former, the results of adsorption could be highly improved by using it in a *statu nascendi*. After the adsorption, serum albumin and pseudoglobulin lost their solubility in water, and the salt solubility of globulin was decreased. These changes meant that a denaturation of the proteins had occurred. Adsorbed proteins went into solution if treated with diluted alkaline solutions (to some extent also, by using acids) in a way similar to that by which adsorbed enzymes were eluted by Willstätter and his co-workers. Under the same conditions, the elution gave best results (practically complete) in serum albumin, while only 40 per cent of the adsorbed euglobulin could be eluted, pseudoglobulin having come between those two. It was demonstrated, by remov-

ing the alkali by dialysis and electrodialysis, that the eluates of adsorbed serum albumin and pseudoglobulin always to some extent contain water-soluble, heat-coagulable protein. The amount varied with the relation of protein and alkali, between 26 and 59 per cent. It was supposed that this water-soluble protein was formed from the adsorbed one by reversal of the denaturation, as similar occurrences had been described by Spiegel-Adolf in heat and alcohol denaturation of some proteins of the serum, and were missed by her in changes due to the effect of short wave light and rays. It is suggested that reversibility of protein denaturation occurs in cases in which no primary chemical changes take place. As the use of adsorption and elution of toxins and antibodies for their purification has been increasing in the last years, the studies mentioned above may be of some practical value.

<div align="center">

ULTRAFILTRATION

II. BOUND WATER (HYDRATION) OF BIOLOGICAL COLLOIDS

By DAVID M. GREENBERG

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(*From the Division of Biochemistry, University of California Medical School, Berkeley*)

If the definition is accepted that bound water loses its solvent properties, then in colloidal solutions it should be possible to determine the bound water of the colloid by ultrafiltration experiments, with the use of an appropriate reference substance. On this conception, if there is an appreciable amount of bound water in the system, the concentration of the reference substance in the ultrafiltered liquid will be higher than the concentration of the reference substance with respect to the total water of the colloidal solution. From the concentration differences, the amount of bound water is readily calculable.

Experiments to determine the bound water of a number of colloids from biological sources, namely blood serum, gelatin, casein, starch, and pectin, were carried out by the ultrafiltration method, urea being used for the reference substance. The experiments uniformly gave the result that within the error of the method of analysis for urea *there is no detectable amount of bound water associated with any of the colloids that were tested.*

This finding leads to the conclusion that those theories are in-

correct which attempt to explain the physical properties and stability of hydrophylic colloids on the basis of high degrees of hydration.

FURTHER STUDIES OF THE ADRENAL CORTICAL HORMONE

By J. J. PFIFFNER, HARRY M. VARS, P. A. BOTT, AND
W. W. SWINGLE

(*From the Laboratory of Biology, Princeton University, Princeton*)

A quantitative study has been made of the extraction and further fractionation of the adrenal cortical hormone.

Method of Assay—Additional evidence is presented upon the adequacy of the assay technique previously described.[12] The effect of rapid reduction of dosage to a predetermined minimum has been studied.

Yield of Cortical Hormone—Further reextraction of the cortical tissue residues after the routine procedure of extraction showed that less than 5 per cent of additional hormone could be recovered. Extracts made from whole glands showed 5 to 10 times more potency than extracts made from dissected cortex alone (based on equivalent weights of tissue). Extracts of approximately equal potency but lower solid content were obtained by using the same fractionation procedure as previously described but decreasing by 50 per cent the thoroughness of extraction of the respective fractions. In this simplified technique the glands were extracted once with alcohol for 48 hours, the benzene-soluble fraction was extracted twice with acetone, the acetone-soluble fraction was distributed twice between 70 per cent alcohol and petroleum ether, and the alcohol-soluble fraction was filtered only once through permutit. Freezing the whole adrenal glands for a period of 1 month had no effect upon the yield of hormone. Extracts preserved with benzoic acid at 5° retained their full activity for periods of 3 to 5 months. Longer periods have not been studied.

Further Fractionation—Experiments upon the distribution of the activity between immiscible solvents and acid and alkaline solutions have been made. Our most active preparation was ob-

[12] Harrop, G. A., Pfiffner, J. J., Weinstein, A., and Swingle, W. W., *Proc. Soc. Exp. Biol. and Med.*, **29**, 449 (1932).

tained by hexane fractionation. The minimum daily kilo dose of this fraction necessary to maintain adrenalectomized dogs in normal physiological condition was found to be about 0.02 mg., representing 0.5 gm. of whole beef adrenal gland.

THE DISTRIBUTION OF ANDROTIN (MALE SEX HORMONE) IN THE MALE

By D. ROY McCULLAGH

(From the Cleveland Clinic Foundation, Cleveland)

A technique has been developed for the study of testicular function. The hormone which causes comb growth in capons (androtin) is extracted from the body fluids and assayed biologically. Methods of extraction and assay have been developed which are sufficiently simple to make extensive clinical investigations possible.

The urine and the spinal fluid are extracted with chloroform by a method based on the procedure of Funk and his collaborators. The hormone is extracted with ether from blood, desiccated by mixing with anhydrous sodium sulfate. After removing the extractive, the androtin is taken up in oil and injected without further purification, since the processes of concentration and purification are potential sources of error.

The amount of androtin in blood and urine varies considerably with age, reaching a maximum value during early adult life. The amount of hormone in the blood and urine of normal individuals of the same age is also definitely variable. The body fluids of eunuchs do not contain a demonstrable amount of androtin. The concentration of the hormone in the blood is much greater than in the spinal fluid or in the urine. However, there appears to be a direct relationship between the amount of hormone in the blood and the amount secreted by the kidneys.

No definite threshold has been established; it appears that whenever there is a demonstrable amount of androtin in the blood, the hormone is excreted in the urine.

REVERSIBLE INACTIVATION OF THE PLASMA CALCIUM-INCREAS-ING PRINCIPLE OF BOVINE PARATHYROID GLANDS*

BY WILBUR R. TWEEDY AND MASAMICHI TORIGOE

(From the Department of Physiological Chemistry, Loyola University School of Medicine, Chicago)

It appears that parathyroid hormone, extracted by the senior author's method of preparation,[13] may be completely inactivated when allowed to remain in contact with a 40 per cent aqueous solution of formalin at room temperature for periods of 1 to several hours. Partial reactivation of such products has been successfully produced by boiling in 0.001 N hydrochloric acid for 20 minutes.

Parathyroid hormone has also been completely inactivated, when suspended in absolute methyl alcohol containing 0.1 N HCl and allowed to remain at room temperature for 24 hours. The inactivated hormone is partially reactivated by standing in contact with 0.08 N sodium hydroxide at 0° for 24 hours.

Inactivation has been produced by various mixtures of pyridine and acetic anhydride, but an adequate method of reactivation has not been developed.

THE CHEMICAL STUDY OF INSULIN

BY H. JENSEN AND E. A. EVANS,

(From the Department of Pharmacology, the Johns Hopkins University, Baltimore)

Research on the chemical constitution of insulin has been continued in two directions. First, the various amino acids resulting from the hydrolysis of insulin have been determined. This further allows the identification of any constituents physiologically important in the action of insulin and chemically different from the known amino acids. Secondly, by treating the insulin molecule with chemical reagents acting definitely on certain groupings to ascertain the importance of such groups for the hypoglycemic effect of the hormone.

[13] Tweedy, W. R., J. Biol. Chem., 88, 649 (1930). Tweedy, W. R., and Smullen, J. J., J. Biol. Chem., 92, p. lv (1931).

The hydrolysis of insulin has been undertaken anew with Dr. Wintersteiner of Columbia University. Besides the amino acids, cystine, tyrosine, arginine, histidine, lysine, and leucine, which have been previously obtained from insulin, glutamic acid has now been isolated. No evidence for the presence of another dibasic amino acid has been obtained. Judging by the amount of ammonia nitrogen (9.58 per cent of the total nitrogen) found in the determination of the nitrogen distribution in insulin, a comparatively large amount of glutamic acid should be present. Leucine seems to be present in rather large amounts (25 to 30 per cent). Amino acids, other than those mentioned, have not been found as yet, and are present, if at all, in only very small amounts. The presence of proline and hydroxyvaline is doubtful. No evidence of the presence of a constituent differing in its composition from an amino acid has been obtained.

If one does not assume that the insulin molecule as a whole is responsible for the physiological action, one may attribute the hypoglycemic effect of the hormone to a grouping of certain component amino acids embedded in the molecule. All attempts to separate such active components from the insulin molecule have thus far failed. The simultaneous occurrence of cystine and glutamic acid suggests a combination of these amino acids similar to that encountered in glutathione, which may be essential for the action of the hormone and which may have the chemical structures shown in the accompanying formulæ:
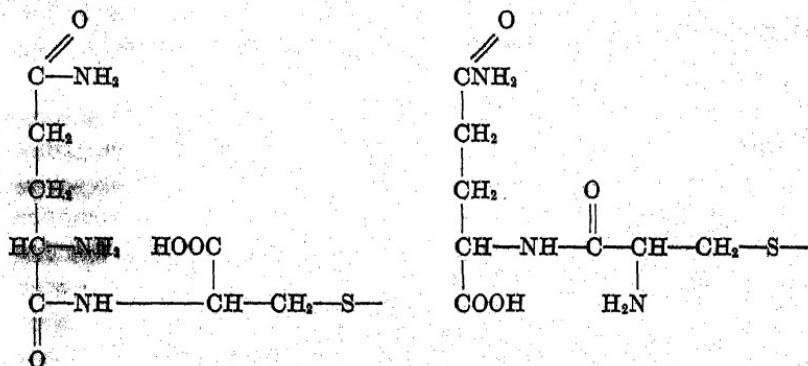
The fact that insulin cannot be inactivated without removing part of the sulfur seems to indicate that cystine plays an important

rôle in the action of the hormone. Exact proof that the glutamic acid is linked to cystine as indicated in the accompanying formulæ cannot be furnished at the present time. These structures are proposed only as a working hypothesis on which to base further efforts towards the elucidation of a possible specific constituent of insulin.

The action of acid and alkali on insulin has been studied in more detail. The results thus far obtained seem to support the postulation of the above grouping in an insulin molecule. This hypothesis is proposed in only the most cautious and tentative manner until further data, either supporting or opposing the postulated structures, are available.

Experiments are now in progress to obtain further proof of the presence of a specific constituent in the insulin molecule, and an attempt is also being made to synthesize cystine peptides having the postulated structures.

THE EFFECT OF INSULIN ON THE BLOOD DIASTASE

By E. REID

(From the Department of Biochemistry, School of Medicine, Western Reserve University, Cleveland)

A study has been made of the diastatic activity of the blood, liver, and muscle in man, and in the dog, rabbit, and rat, with the idea of securing further information on the function of the diastatic ferment in carbohydrate metabolism. The method of Myers and Killian[14] was employed. The findings to date permit the following conclusions.

The blood diastase in diabetics, untreated with insulin, is almost invariably higher than that of normal individuals. Diabetics receiving insulin, on the other hand, usually show a normal or even subnormal blood diastase.

There is a decrease in the blood diastase of dogs for the first few days after pancreatectomy, but if the animals are kept alive with insulin, the blood diastase rises to a point higher than the preoperative level. When insulin injections are discontinued, the blood diastase again falls slightly. It is noteworthy that the diastase in the blood after pancreatectomy never entirely disappears and

[14] Myers, V. C., and Killian, J. A., J. Biol. Chem., **29**, 179 (1917).

that, therefore, the pancreas cannot be the only source of this enzyme. In a normal dog, the fall in blood diastase after insulin injection is accompanied by an increase in the liver diastase.

In normal rabbits, insulin lowers the blood diastase. If sufficient insulin is administered to produce hypoglycemic convulsions, the blood diastase first falls, then rises sharply when convulsions take place.

It would not appear that diastase has any function in the blood. It is suggested that it is only when it is recalled into the liver cells (possibly by the action of insulin) that it is enabled to exert its enzymatic function.

PROTECTION OF INSULIN BY ANTITRYPSIN

By BEN K. HARNED AND THOMAS P. NASH, JR.

(From the Department of Chemistry, University of Tennessee College of Medicine, Memphis)

Antitrypsin concentrates prepared from the roundworm of swine, *Ascaris lumbricoides*, protect insulin from inactivation by pancreatin mixtures under conditions optimal for protein digestion. At pH 8, and with an initial concentration of 6 units of insulin per 1.0 cc. of the final mixture, the pancreatin employed inactivates the insulin so promptly at room temperature that injections of 1.0 cc. quantities immediately after mixing are without effect upon the blood sugar values of fasted, 2 kilo rabbits. Incubation of the pancreatin-isulin mixtures at 37.5° produces increases of non-protein nitrogen indicating digestion of the insulin. When antitrypsin is included in the pancreatin-insulin digests, there is no significant increase in the non-protein nitrogen values, even after 4.5 hours incubation at 37.5°; isoelectric insulin may be precipitated from the incubated digest, and the rabbit assay shows that 90 per cent of the initial insulin potency remains.

THE EFFECT OF ANTERIOR PITUITARY EXTRACTS ON THE NITROGEN BALANCE AND URINE VOLUME OF DOGS

By OLIVER HENRY GAEBLER

(From the Department of Laboratories, Henry Ford Hospital, Detroit)

A series of experiments was carried out in which extracts of anterior pituitary glands (beef), prepared by a modification of the

method of Teel, were administered intraperitoneally or subcutaneously to dogs kept on a constant diet.   Three animals were studied for a period of about 3 months, with daily urine analyses.   The results were similar to those reported by Teel and his coworkers. The effect of the extracts on the nitrogen balance was striking. The total nitrogen excreted daily in the urine decreased 1.5 to 5.1 gm., a low level of excretion persisting for 2 to 3 days after injection.   No change in the feces nitrogen was found, and the blood non-protein nitrogen was lowered; hence the fall in urine nitrogen was apparently not due to an effect on absorption or to a renal effect.   Subcutaneous injections of 50 cc. of the extract on each of 2 successive days resulted in an enormous increase in urine volume, 2 to 4 liters of urine being voided daily by a 20 kilo bitch during the height of the action.   Concomitant findings were extreme thirst and slight rise in temperature.   In a single instance injection was followed by lactation, which persisted for about 4 days.   Control injections of boiled extract and other solutions yielded negative results throughout.

Whether the effect on the nitrogen balance is to be ascribed to a growth hormone in the extracts is not entirely clear, since in some animals the nitrogen stored is subsequently lost again.   The effect of the extracts on the nitrogen balance is greatest on high protein diets.

The extracts were obtained from the Research Division, Parke, Davis and Company, through the courtesy of Dr. Kamm and Dr. Bugbee.

PREPARATION, PURIFICATION, AND ASSAY OF AN ANTERIOR PITUITARY-LIKE SUBSTANCE FROM URINE DURING PREGNANCY

By P. A. KATZMAN and EDWARD A. DOISY

(*From the Laboratory of Biological Chemistry, St. Louis University School of Medicine, St. Louis*)

A method based on the adsorption of the anterior pituitary-like substance from urine during pregnancy on finely divided benzoic acid has yielded satisfactory extracts.   By this procedure an average of 60 to 70 per cent of the original activity can be recovered.   The potency is about 170 mouse units per mg.

By a single repetition of this procedure and further purification by fractional precipitation with acetone, the potency has been increased to 3800 mouse units per mg. These purified preparations give the Millon and biuret reactions. A procedure, not yet adequately standardized, has enabled us to increase the potency to over 5000 mouse units per mg. without eliminating the biuret reaction.

The assay of these preparations is based on the induction of premature opening of the vagina and estrus in immature mice and rats. This serves as an adequate basis for assay since the preparations are free from estrogenic material. As much as 1675 mouse units failed to induce estrus in spayed rats. These extracts are more active in mice than in rats, to such an extent that 1 rat unit is equivalent to 4 mouse units. This ratio (1:4) was the same for extracts prepared by the benzoic acid method as well as for those prepared by adsorption on charcoal and elution with phenol. These findings do not agree with Zondek's results but may be due to the different methods of assay used.

Histological studies made by Dr. W. D. Collier have shown that the effect of the administration of small doses of this pituitary-like hormone upon the ovaries of immature rats and mice examined 1 and 2 days following the premature opening of the vagina and estrus was stimulation of a greater number of follicles to development with ovulation and formation of normal corpora lutea of ovulation. Apparently no abnormal process, either of luteinization or otherwise, occurs. The effect induced by these experimental conditions during the first few days after estrus is entirely a quantitative speeding up of the normal process.

THE PREPARATION AND BIOASSAY OF THEELOL

By SIDNEY A. THAYER and D. W. MacCORQUODALE

(*From the Laboratory of Biological Chemistry, St. Louis University School of Medicine, St. Louis*)

Owing to certain difficulties in the preparation of theelol the procedure has been studied in detail. After extraction of the theelin from 0.5 NaOH with ethyl ether, the theelol can be extracted with butyl alcohol. A new principle introduced in the

purification of theelol is the concentration of an alkaline (NaOH) solution to an appropriate volume. The sodium salt precipitates but the tarry materials that are otherwise difficult to remove remain in solution.

Though we have attempted in various ways to ascertain whether theelin contaminates our purified theelol, our results are entirely negative. The potency of theelol was not altered by the use of hydroxylamine to make the oxime of any theelin which might have been present. It was also impossible to obtain a semicarbazone of theelin from the theelol preparations.

The bioassay of theelol shows an enormous difference in the number of units per mg., depending upon whether a partial or full estrus smear is taken as a positive response; with theelin the difference is small.

Different preparations of theelin which had been kept for periods varying from 1 week to 8 months assayed more than 50 million mouse units per gm. by the Marrian-Parkes procedure.

COMPARISON OF THEELIN AND THEELOL WITH EXTRACTS OF LIQUOR FOLLICULI

By JACK M. CURTIS

(*From the Laboratory of Biological Chemistry, St. Louis University School of Medicine, St. Louis*)

Extracts of liquor folliculi are prepared by treating fresh follicular fluid with alcohol. These extracts are purified by partitions between water and ethyl ether, aqueous alcohol and petroleum ether, alkaline solutions and ether, and aqueous alcohol and benzene. The product is distilled under a pressure of 0.016 mm. of Hg at a temperature of 140–160°, the distillate being precipitated from alcohol by addition of water.

The solubility and partition relationships of these extracts were compared to those of theelin and theelol. The relation of the mouse unit to the rat unit of all three materials as well as the response of the injected ovariectomized rats to these materials has been studied. Indirect evidence obtained in this way leads to the belief that the active material in the ovary is theelin.

CHANGES IN THE COMPOSITION OF THE BLOOD IN THE RABBIT DURING PREGNANCY AND LACTATION

By MARTHA E. DAVIS AND MEYER BODANSKY

(From the John Sealy Memorial Research Laboratory and the University of Texas School of Medicine, Galveston)

The present observations are based on fifteen pregnancies in eleven individuals and on eleven parallel control periods in seven animals.

During the first 10 days of pregnancy, the serum protein level remained normal (average of 6.3 to 6.4 gm. per 100 cc.). This was followed by a gradual fall to lower levels. The concentration during the 5 days preceding delivery averaged 5.3 gm. In all the pregnancies, the lowest values were obtained within 12 hours after delivery (4.2 to 5.4 gm., with an average of less than 5 gm.). After this, recovery was rapid, but usually the normal serum protein level was not attained during the 25 day period of lactation.

The serum calcium averaged 15.85 mg. per 100 cc. during the first 20 days of pregnancy, as compared with 15.3 mg. obtained for the controls. A sharp decrease occurred in the pregnant group during the next 10 days, the average for this period being 14.5 mg. In general, the lowest values were observed within 12 hours post partum (average of about 14 mg.), after which a moderate rise occurred, but the highest level attained during the period of lactation was on an average 1 mg. below the normal controls. In the limited number of observations on inorganic phosphorus, the lowest values were likewise encountered at the time of delivery.

The maximum variation for potassium in the controls was 14 to 20 mg. with an average of 18.3 mg. The average for the pregnant group was somewhat higher and the range of variability somewhat greater (14 to 23 mg.). With the onset of lactation and persisting throughout the 25 day period, the average potassium was 20 mg., the range of values being 16.4 to 22.7 mg.

During both gestation and lactation the ratio of urea to non-protein nitrogen tended to be lower than in the controls. No consistent change in the absolute amount of urea was noted, however, during pregnancy, but during lactation this tended to be be somewhat below the normal average.

It appears from a preliminary study of the problem that the changes in the composition of the blood which occur in the rabbit during pregnancy and lactation are consistent and that the data may be clearly differentiated from the normal ranges of variability.

THE EFFECT OF THE INGESTION OF SODIUM, POTASSIUM, AND AMMONIUM CHLORIDES

By FRANK H. WILEY, LEONA L. WILEY, and DOROTHY S. WALLER

(*From the Department of Internal Medicine, Medical School, University of Michigan, Ann Arbor*)

A normal individual on a maintenance diet, low in ash, was fed 100 milli-equivalents each of sodium chloride, potassium chloride, and ammonium chloride daily. The urine was analyzed daily for sodium, potassium, calcium, magnesium, chlorides, inorganic sulfates and phosphates, nitrogen, ammonia, titratable acidity, and total solids. The stools were examined in 3 day periods for sodium, potassium, calcium, magnesium, total phosphates, total sulfur, and chlorides.

The ingestion of sodium chloride over a period of 9 days was accompanied by a slight retention of sodium and an increased excretion of potassium and calcium in the urine. There was a preliminary retention of chlorine but balance was established before the end of the period. There were no marked changes in the components of the stool.

The ingestion of potassium chloride was accompanied by an increased excretion of sodium in the urine. A lag in the chlorine excretion was observed but, as in the case of sodium chloride, balance was obtained in the 9 day period. There were no other noticeable changes.

The ingestion of ammonium chloride caused an increased excretion of sodium, potassium, calcium, magnesium, ammonia, inorganic sulfates, and inorganic phosphates in the urine. The potassium, calcium, and magnesium of the stools decreased. The titratable acidity of the urine was markedly increased. In the control period following the ingestion of ammonium chloride there was a retention of sodium, potassium, calcium, and magnesium.

AN ECONOMY OF WATER IN RENAL FUNCTION REFERABLE TO UREA

By JAMES L. GAMBLE, C. F. McKHANN, AND A. M. BUTLER

(From the Department of Pediatrics, Harvard Medical School, Boston)

The plan of the experiments consisted in placing rats on a basal maintenance diet containing a minimal amount of protein and inorganic elements and then adding to this diet materials which must enter the urine. Water intake was unrestricted and the experiments are regarded as describing the optimal water requirement for removal in urine of the substances studied. It was found that, when sodium chloride and urea were added together to the basal diet, the sum of their concentrations in the urine was much higher than an expected value calculated from measurements of the concentrations of sodium chloride and of urea when added singly to the basal diet. A considerable economy of water in the removal of mixtures of the two substances in urine is thus described. The excretion of several other non-electrolytes, *viz.* glucose, galactose, and creatinine, was then studied, separately and when fed with sodium chloride, and it was found that the water requirements for the removal of these substances remain additive when they enter the urine along with salt.

THE ACID-BASE EQUILIBRIUM AND PHOSPHORUS METABOLISM IN HYPERTHERMIA

By CORNELIUS A. DALY AND ARTHUR KNUDSON

(From the Department of Biochemistry, Medical Department of Union University, Albany Medical College, Albany)

The experiments were carried out on dogs, heated either by radiothermy or hot water. Both types of heating gave similar results. Changes in the acid-base equilibrium were followed by determining serum pH, $BHCO_3$, and pCO_2, together with urine pH and NH_3. The phosphorus metabolism was studied by determining inorganic phosphorus on whole blood, serum, and urine, acid-soluble phosphorus on whole blood, plasma, and cells, lipoid phosphorus on plasma and cells, and total phosphorus on whole blood, plasma, cells, and urine.

Our experiments indicate that an alkalosis, no change in pH,

or an acidosis may be produced by hyperthermia, depending upon the length of exposure and maximum increase in temperature. We have formulated, for these varying pH values, an explanation which is similar to the one given by Koehler[15] and his coworkers for acid-base changes in anoxemia. When the loss of CO_2 is greater than the production of CO_2 and lactic acid, an alkalosis results. No change in pH is found if the loss of CO_2 just compensates the production of acids. Finally an acidosis is produced when the loss of CO_2 is less than the formation of CO_2 and lactic acid. We were unable to confirm Bischoff's[16] suggestion that during alkalosis the acid-soluble phosphorus increased as the inorganic fraction fell, according to the Lawaczek reaction. We found that each of the phosphorus fractions decreased in both whole blood and plasma. The phosphorus in the cells showed a slight rise, but this seems to be only an apparent change since the cells shrink to some extent as the result of a water loss during heating.

ALKALOSIS AND THE CAPACITY FOR WORK

By D. B. DILL, H. T. EDWARDS, and J. H. TALBOTT

(*From the Fatigue Laboratory, Morgan Hall, Harvard University, Boston*)

Some time ago we suggested that the highest attainable oxygen debt might be increased by an initial state of alkalosis. Experiment shows such an effect but it is no more than one-half as great as our calculations indicated. Thus, a runner in an alkaline state ran 6 minutes and 4 seconds to exhaustion in comparison with 5 minutes, 22 seconds starting from a normal state. The oxygen debt was about 20 per cent greater in the first case, although the lactic acid concentration in blood from the femoral vein $3\frac{1}{2}$ minutes after work stopped was 40 per cent greater (0.190 and 0.134 per cent respectively) and the change in alkaline reserve of blood was also 40 per cent greater.

Evidently the appearance and the removal of lactic acid in the blood give an imperfect picture of changes in the muscle. Moderate exercise may cause no increase in blood lactic acid although it may entail an oxygen debt of 2 liters. During the first 5 minutes

[15] Koehler, A. E., Brunquist, E. H., and Loevenhart, A. S., *Am. J. Physiol.*, **63**, 404 (1923).

[16] Bischoff, F., Maxwell, L. C., and Hill, E., *J. Biol. Chem.*, **90**, 331 (1931).

of recovery after running to complete exhaustion, on the other hand, samples of blood from the femoral vein show little decrease in the level of lactic acid. During this period little lactic acid leaves the muscle, for samples of blood from an artery have about the same lactic acid content as simultaneous samples from the femoral vein. Notwithstanding these facts the oxygen debt is nearly one-half paid during the first 5 minutes of recovery.

While several explanations can be advanced, the following is suggested as most plausible. In the first stage of recovery resynthesis of phosphocreatine is of major importance. While this is going on, there is little net resynthesis of lactic acid to its precursor and little evidence in the blood of the recovery processes which are going on in the tissues. This stage of recovery requires about 5 minutes. The removal of lactic acid then proceeds at a slow and decreasing rate, requiring from 60 to 90 minutes for completion. An initial alkaline state increases the capacity for neutralizing lactic acid but may have no effect on phosphocreatine breakdown. Thus, if the quantity of lactic acid formed is increased one-half, the oxygen debt may be increased one-fourth or less.

CHANGES IN CERTAIN BLOOD CONSTITUENTS PRODUCED BY PARTIAL INANITION AND MUSCULAR FATIGUE

By FREDERIC W. SCHLUTZ and MINERVA MORSE

(*From the Department of Pediatrics, the University of Chicago, Chicago*)

The effect on certain blood constituents of treadmill exercise and of swimming under conditions of normal diet and of undernutrition has been studied intensively in a young dog.

The observations have been made in 4 week periods extending over 7 months. Analyses of the blood were made four times a day on 10 days of each period. The results to date may be summarized as follows: (1) Treadmill exercise on a normal diet produced a change in the acid-base balance of the blood in the direction of CO_2 deficit. When coupled with undernutrition, the displacement from normal was slightly greater. The return to normal was prompt in both periods. (2) There was a slight increase in serum lactate but no change in serum sugar as a result of the exercise. (3) Swimming produced a marked displacement of the acid-base balance of the blood in the direction of fixed acid ex-

cess which was more marked in the period of undernutrition than in the period of normal food intake. The return to the normal acid-base condition was delayed in the period of undernutrition. (4) There was also a marked increase in serum lactate and serum sugar following swimming, which was more marked in the period of undernutrition.

ACID-BASE PATHS IN HUMANS

By A. BAIRD HASTINGS and N. W. SHOCK

(From the Lasker Foundation for Medical Research and the Department of Medicine, the University of Chicago, Chicago)

With the micro acid-base pipette is has been possible to make frequent observations of the acid-base balance of the blood of humans under conditions of fixed alkali excess, fixed acid excess, CO_2 excess, and CO_2 deficit. As many as twenty-five observations on a single individual have been made in the course of a day. The results of these experiments when plotted on triaxial coordinate paper illustrate the following points. (1) Changes produced by overbreathing and rebreathing are in the direction of the curve of true serum when titrated with carbon dioxide. (2) Changes produced by the ingestion of ammonium chloride and sodium bicarbonate are in a direction approximately at right angles to this curve. (3) There is maximal dissociation of the acid-base changes produced on the one hand by respiratory factors and on the other by non-respiratory factors. (4) Normal variations in the acid-base balance have been established for (a) single individuals during a day, (b) single individuals from day to day, and (c) different individuals.

STUDIES ON THE METABOLISM OF GROWING DOGS

By LEOPOLD R. CERECEDO and JAKOB A. STEKOL

(From the Division of Biochemistry, University of California Medical School, Berkeley)

The main results of this investigation may be summarized as follows:

1. We have been able to raise puppies, which had been weaned when they were 6 weeks old, to maturity on Cowgill's diet to which tomato juice and cod liver oil were added.

2. In 24 hour samples of urine, collected without catheteriza-

tion, we determined total nitrogen, urea, and inorganic, ethereal, and total sulfur. We obtained data covering a metabolic period of 18 days for a dog less than 3 months old.

3. Feeding experiments with isobarbituric acid showed certain differences between adult and growing dogs. Whereas in adult dogs this compound is partly excreted in the urine in the form of urea, in growing dogs the amount of urea excreted remains constant. In adult dogs we found, after feeding isobarbituric acid, a drop and, in some cases, a complete disappearance of the neutral sulfur in the urine. In the growing dog, on the other hand, the excretion of neutral sulfur is not affected at all.

4. In the growing as well as in the adult dog there is a rise in the output of ethereal sulfur on the day following the feeding of isobarbituric acid.

THE CHEMISTRY OF EMBRYONIC GROWTH

III. A BIOCHEMICAL STUDY OF THE EMBRYONIC GROWTH OF THE PIG, WITH SPECIAL REFERENCE TO NITROGENOUS COMPOUNDS

By VERNON A. WILKERSON AND ROSS AIKEN GORTNER

(*From the Division of Agricultural Biochemistry, University of Minnesota, St. Paul*)

A total of 1552 pig embryos, ranging from 4 to 240 mm. in length, were utilized in this study. After dehydration in acetone and extraction with absolute ether and alcohol, the nitrogenous compounds were studied by the Van Slyke nitrogen distribution method. Water content, total sulfur, total ash, glutathione, and tyrosine were also determined.

During development there was a decrease in those nitrogen fractions reacting as arginine and histidine and an increase in lysine, although the total bases remained essentially constant at 38 to 39 per cent of the total nitrogen. Humin nitrogen, amide nitrogen, and cystine remained practically unchanged. Total nitrogen showed an early decrease until the 30 mm. stage and then remained remarkably constant.

Water rapidly decreased in the early stages from 94.67 per cent at the 6 to 7 mm. stage to 91.71 to 91.02 per cent at the 15 to 160 mm. stages, after which there was a second decrease to 90.34 per cent at the 200 mm. stage and 88.7 per cent in the 240 mm.

embryos. Glutathione showed a rapid increase to a peak at the 30 mm. stage, then a rapid decrease at the 40 mm. stage, followed by a slower but continuous fall throughout the later growth stages.

Total sulfur showed an increase to the 50 mm. stage and then a gradual decrease.

Ash presented an initial increase in the very early stages, followed by a slight decline, and then a continued increase as the skeletal structure developed, while tyrosine showed a gradual decline throughout the entire developmental period. The detailed paper will discuss the significance of the changes and their relationship to tumor chemistry and evolutionary processes.

PHOSPHORUS METABOLISM IN MUSCULAR DISEASE

By ERWIN BRAND AND MEYER M. HARRIS

(From the Departments of Chemistry and Internal Medicine, New York State Psychiatric Institute and Hospital, New York)

In several previous publications,[17] the relation of glycine to creatine metabolism, sulfur metabolism, and other metabolic processes was demonstrated in cases of muscular dystrophy.

Some of these findings have been recently confirmed by Thomas, Milhorat, and Techner.[18] They have also reported clinical improvement by the prolonged administration of glycine to certain cases of primary muscular disease. We are undertaking further investigations of this phase of the problem.[19]

Because of the present importance of phosphocreatine for muscle physiology, it may be of interest to report the effect of ammonium chloride feeding upon the creatine and phosphorus excretion in two of the cases of the group which was studied.

One patient, who had a low creatinine and high creatine excretion and who was markedly incapacitated in both upper and lower extremities, showed no increase in phosphorus and creatine elimination upon the prolonged feeding of large amounts of ammonium

[17] Brand, E., Harris, M. M., Sandberg, M., and Ringer, A. I., Am. J. Physiol., 90, 296 (1929). Brand, E., Harris, M. M., Sandberg, M., and Lasker, M. M., J. Biol. Chem., 87, p. ix (1930). Brand, E., and Harris, M. M., J. Biol. Chem., 92, p. lix (1931).

[18] Thomas, K., Milhorat, A. T., and Techner, F., Z. physiol. Chem., 205, 93 (1932). Milhorat, A. T., Techner, F., and Thomas, K., Proc. Soc. Exp. Biol. and Med., 29, 609 (1932).

[19] Aided by a grant from the Chemical Foundation.

chloride. The other patient, with relatively high creatinine and low creatine excretion and with far less muscular disability, showed definite increases in creatine and phosphoric acid excretion during such feeding.

More recently, biochemical studies of biopsy material from a case of familial periodic paralysis[20] showed that the muscle, although appearing normal histologically, still contained reduced amounts of creatine and acid-soluble phosphorus (see Table I).

These studies point to a disturbance of phosphorus metabolism in certain muscular diseases. They also suggest, in agreement with earlier experiments on animals,[21] that the muscle tissue is

TABLE I

Biopsy; gastrocnemius muscle	Acid-soluble P per 100 gm. of muscle				Creatine per 100 gm. muscle
	Total	Or-ganic*	Total inor-ganic	Phos-pho-crea-tine†	
	mg.	*mg.*	*mg.*	*mg.*	*mg.*
Case of familial periodic paralysis, May 26, 1931....................	76	13	63	28	278
Normal case, June 8, 1931...........	143	53	90	34	401

* Pyrophosphate and phosphoric acid esters.

† The figures are too low because the determination could only be carried out by a method similar to that of Ferdmann, D., *Z. physiol. Chem.*, 178, 52 (1928).

probably a source of the increased excretion of phosphorus, resulting from ammonium chloride feeding.

THE EXCRETION OF CALCIUM IN THE URINE OF HEALTHY IN-
FANTS AND CHILDREN

By GENEVIEVE STEARNS

(*From the Department of Pediatrics, State University of Iowa, Iowa City*)

An attempt has been made to determine some of the factors influencing the excretion of calcium in the urine of infants and children given standard diets neutral or alkaline in ash.

[20] Zabriskie, E. G., and Frantz, A. M., *Bull. Neurol. Inst. New York*, 2, 57 (1932).

[21] Goto, K., *J. Biol. Chem.*, 36, 355 (1918).

With a given diet, the 24 hour urinary excretion of calcium varies with the individual. The level of urinary calcium of the individual increases steadily throughout the period of infancy, and presumably throughout childhood.

The intake of calcium *per se* is not an important factor in determining the level of urinary calcium in either infants or children. This is contrary to the findings in adults. No relation could be observed between the retention of calcium and the quantity of urinary calcium excreted by infants or children.

Dietary factors other than calcium and acid-base relationships influence, at least temporarily, the level of urinary excretion of calcium. Of these factors the mineral relationships of the diet are probably important.

SUCCESSIVE MINERAL BALANCES IN CHILDHOOD

By HELEN A. HUNSCHER, FRANCIS COPE, ALICE NOLL,
AND ICIE G. MACY

(From the Research Laboratory of the Children's Fund of Michigan, Detroit)

AND THOMAS B. COOLEY, GROVER C. PENBERTHY, AND
LILLIAN ARMSTRONG

(From the Children's Hospital of Michigan, Detroit)

Two girls, 7 and 11 years of age and weighing 23 and 30 kilos respectively, have been maintained on a constant food intake, and the total acid-base balance determined over four and five successive 5 day balance periods in order to secure data on the metabolic response of the growing child to an adequate unchanging diet.

The basic elements (Na, K, Ca, and Mg) and the acid elements (Cl, S, and P) have been determined in the food, urine, and feces respectively by standard methods of chemical analyses, and, for direct comparison the amount of each substance has been calculated in terms of 0.1 N acid or alkali. A positive base retention was found in all nine acid-base balances. Ca, Mg, K, P, and S were all stored over the periods of observation, whereas negative balances in Na, Cl, and N occurred in the first periods of adjustment but later became positive.

The interrelationship of the various elements to each other and to the metabolism of nitrogen and fat, together with the nitrogen partition of the urine under a constant dietary intake, will be

reported in detail later not only on the present subjects but on a group of active children living under an unusually healthful and wholesome environment and participating in their usual school activities.

NORMAL RANGE OF SERUM CALCIUM AND INORGANIC PHOSPHORUS IN WOMEN

By JAMES W. MULL and ARTHUR H. BILL

(*From the Research Laboratory, Maternity Hospital, and the Department of Obstetrics, School of Medicine, Western Reserve University, Cleveland*)

Serum Ca of 205 normal, non-pregnant women was determined by a modified Kramer-Tisdall method and inorganic P by the method of Kuttner and Cohen. Grouping by diet and by age shows that the serum Ca of adult women is not affected by age or diet, so long as the intake is adequate. A distribution curve shows that over 72 per cent of the 205 fall within ±0.4 mg. of the average, 10.6 mg. per 100 cc. The P, however, does vary with age. The average shows a decline at approximately 30 years. 80 per cent of the younger women come within ±0.4 mg. of 3.8 mg., while 67 per cent of the older group are within ±0.5 mg. of 3.3 mg. Two or more determinations on the same individual at extended intervals show a mean deviation from the individual's average of only 0.3 mg. for Ca and 0.22 mg. for P. There was some indication of a fall in Ca during January, February, and March, although, in the main, the evidence was inconclusive. No seasonal variation was observed in P, but the fall following the ingestion of food was confirmed.

PHOSPHORUS AND CARBOHYDRATE METABOLISM IN THE AUTOLYZING MUSCLE OF NORMAL, HYPERTHYROID, AND ADRENALECTOMIZED ANIMALS

By MARY V. BUELL and MARGARET B. STRAUSS

(*From the Heart Station and Chemical Division of the Department of Medicine, the Johns Hopkins Hospital, Baltimore*)

The known P compounds were studied quantitatively and correlated with the process of glycolysis in the autolyzing muscle tissue of normal, hyperthyroid, and adrenalectomized animals. The determined total acid-soluble P in resting normal cat and

rabbit gastrocnemii was, within the limits of experimental error, found to be the sum of all the known acid-soluble P compounds; namely, phosphocreatine, orthoinorganic phosphate, adenylic pyrophosphate, Embden ester, and traces of free adenylic acid.

The initial rate of formation of lactic acid and inorganic phosphate was identical. Therefore, if both constituents originated exclusively from a common mother substance, a hexosediphosphate must have been the precursor.

In both hyperthyroid and adrenalectomized cats the distribution of the acid-soluble P of muscle remained approximately normal. The striking variation was found in the lactic acid curve which was characterized by a low initial lactic acid value, and a decreased rate and early cessation of lactic acid production. Also the glycogen was low, a fact which we have shown not to be the cause of the decreased lactic acid production.

These findings suggest the possibility that failure of normal lactic acid production may be the cause of the myasthenia characteristic of hyperthyroidism and of Addison's disease.

EXPERIMENTAL FACTORS INFLUENCING BLOOD PHOSPHATASE VALUES

By AARON BODANSKY, HENRY L. JAFFE, and JOSEPH P. CHANDLER

(From the Laboratory Division, Hospital for Joint Diseases, New York)

Increased serum (or plasma) phosphatase is associated with bone diseases like rickets, von Recklinghausen's disease, Paget's disease, etc.[22, 23]   Its clinical significance is particularly obvious in a curable condition like rickets (serum phosphatase as high as 160 units per 100 cc.) in which the healing is accompanied by a return to normal values (5 to 15 units).[23]   However, although broadly speaking there is a correlation between increased serum phosphatase and bone disease, other factors may be distinguished, particularly when bone lesions are produced experimentally.

Anorexia and Malnutrition—When parathormone or deficient diets are administered, the non-specific effects of eventual anorexia and malnutrition must be distinguished from the specific effects of

[22] Kay, H. D., *J. Biol. Chem.*, **89**, 249 (1930).
[23] To be published.

the treatment. Malnutrition, however caused, lowers plasma and serum phosphatase.[24]

Diet—In rats plasma and serum phosphatase are almost twice as high on a meat diet with an adequate content of calcium and accessory factors as on an adequate stock diet. (All tests were performed during the postabsorptive period.[24]) Furthermore, specific effects on plasma or serum phosphatase may be found during the period of digestion and absorption.[23]

Calcium Deprivation and Ammonium Chloride Acidosis with Decalcification—Marked bone resorption occurs in 3 months old puppies fed a low calcium diet, or a low calcium diet plus ammonium chloride. Ammonium chloride causes osteoporosis even on a diet adequate as to calcium; associated with this osteoporosis is found an increase of serum phosphatase which is a reversal of the usual downward trend observed in control animals during a similar period. In a 6 months old litter osteoporosis and increased serum phosphatase are found on a low calcium diet with or without ammonium chloride but not on an adequate calcium diet with ammonium chloride. In older dogs, neither osteoporosis nor serum phosphatase rise is produced by ammonium chloride on an adequate diet. Low calcium osteoporosis is accompanied by the abolition of the downward phosphatase trend or by a trend toward a higher phosphatase.

The increase of serum phosphatase in growing animals is all the more significant when it occurs in spite of the normal downward tendency observed in such animals over a period of 2 to 3 months, and in spite of anorexia and malnutrition.

RELATIONSHIPS BETWEEN THE CONSTITUTION OF ORGANIC COMPOUNDS AND THEIR EFFECTS UPON ESTERASE AND LIPASE

By DAVID GLICK

(*From the Department of Chemistry, University of Pittsburgh, Pittsburgh*)

Relationships between the structure of organic compounds and their inhibiting effects upon liver esterase have been further demonstrated. It has been found that compounds which act as

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[24] Bodansky, A., and Jaffe, H. L., *Proc. Soc. Exp. Biol. and Med.*, **29**, 199 (1931).

inhibitors upon liver esterase activate pancreatic lipase. This activation has been measured for a number of compounds, and a relationship between their structure and activating effect is shown. A theory is proposed to explain the difference in the effects of the compounds studied upon the two types of enzymes. The compounds investigated include the bile salts.

THE INFLUENCE OF OPTICAL ACTIVITY ON THE PRODUCTION OF KYNURENIC ACID

By CLARENCE P. BERG

(*From the Biochemical Laboratory, State University of Iowa, Iowa City*)

Optical activity determinations have shown that acetyltryptophane prepared as directed in kynurenic acid studies reported previously from this laboratory is racemic. By altering the conditions of synthesis an optically active product has been prepared which, on careful hydrolysis, can be reconverted into free tryptophane exhibiting very nearly, if not quite, the same rotation as the original free acid.

Data bearing upon the use of this product show that it gives rise to an output of kynurenic acid approximately twice as great as that obtained from *dl*-acetyltryptophane, but considerably smaller than the yield from an equivalent amonnt of free *l*-tryptophane. Free *dl*-tryptophane causes an output of kynurenic acid approximately half as great as does the same amount of free *l*-tryptophane. These observations suggest that only the levo component can undergo conversion to kynurenic acid.

In order to answer this question definitely, the resolution of *dl*-tryptophane has been undertaken. The acetyl derivative of *d*-tryptophane has been obtained by preparing *dl*-acetyltryptophane, forming the quinine salt, recrystallizing from methyl alcohol (in which the acetyl-*d*-tryptophane quinine diastereoisomer is the less soluble) to constant rotation, displacing the quinine with alkali, and finally acidifying the alkaline solution to precipitate the derivative. Upon careful hydrolysis of this product with acid, *d*-tryptophane may be liberated. Tests made thus far show little or no kynurenic acid production from either the free or the acetylated *d*-tryptophane. Further studies are in progress.

THE CONJUGATION OF HYDROXYBENZOIC ACIDS IN THE DOG AND IN MAN

By ARMAND J. QUICK

(From the Department of Surgical Research, Cornell University Medical College, New York City)

It was found that when *p*-hydroxybenzoic acid is fed to a dog, it is conjugated with glycuronic acid both through the hydroxyl and the carboxyl groups. By means of extracting the urine with butyl alcohol, it was possible to obtain this diglycuronic acid compound of *p*-hydroxybenzoic acid in pure crystalline form. The glycuronic acid attached to the carboxyl group is readily split off by alkali, while the second molecule of glycuronic acid that is combined in glucoside linkage to the hydroxyl group is hydrolyzed off only after boiling with 1.0 N hydrochloric acid. Although it has not been possible to isolate a diglycuronic acid derivative of the *m*- and *o*-hydroxybenzoic acids in crystalline form, evidence has been obtained that they are similarly formed.

The conjugation of the three hydroxybenzoic acids in man after the ingestion of 3.5 gm. of the acid as the sodium salt is as follows:

Time	Excretion of					
	p-Hydroxybenzoic acid		*m*-Hydroxybenzoic acid		*o*-Hydroxybenzoic acid	
	Free	With glycine	Free	With glycine	Free	With glycine
hrs.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
1	0.44	0.27	0.12	0.50	0.16	0.02
2	0.58	0.54	0.12	0.67	0.19	0.02
3	0.29	0.42	0.01	0.69	0.12	0.02
4	0.00	0.29	0.03	0.33	0.13	0.02

THE EXCRETION OF METHYL URIC ACIDS AFTER THE INGESTION OF METHYLATED XANTHINES

By R. F. HANZAL and VICTOR C. MYERS

(From the Department of Biochemistry, School of Medicine, Western Reserve University, Cleveland)

The effect of the methylated xanthines, theophylline, theobromine, and caffeine, upon the uric acid excretion in the Dalmatian coach dog has been studied and found to be essentially the same

as in man. There was practically no change in the quantity of uric acid excreted after theobromine, but a marked increase occurred after both theophylline and caffeine.

The solubility of several methylated uric acids has been studied and found to be much higher than uric acid. At 17.5° the solubilities were as follows: 1, 3, 7-trimethyl uric acid, 1 part in 260 parts of water, 1, 7-dimethyl uric acid, 1 part in 1400 parts of water, 1, 3-dimethyl uric acid, 1 part in 1930 parts of water, 3, 7-dimethyl uric acid, 1 part in 3350 parts of water, 1-monomethyl uric acid, 1 part in 3530 parts of water, 3, 9-dimethyl uric acid, 1 part in 7380 parts of water, 3-monomethyl uric acid, 1 part in 19,700 parts of water. These differences in solubility between uric acid and the methylated uric acids were made use of in a separation of the uric acid excreted after the ingestion of the methylated xanthines. A microscopic examination of the crystals so obtained showed evidence of the excretion of 1, 3-dimethyl uric acid after the administration of theophylline, and 3, 7-dimethyl uric acid after theobromine. This is in perfect agreement with the colorimetric determination of uric acid in the urine after the feeding of these two xanthines, since 3, 7-dimethyl uric acid does not give a color reaction with the uric acid reagent, while 1, 3-dimethyl uric acid does.

The increase in uric acid excretion after the ingestion of methylated uric acids, except those with a methyl radical in position (7), is proportional to the amount of color they develop with the uric acid reagent. It was found that there was little, if any, demethylation of the methyl uric acids having the methyl radical in position (7) when they were administered orally to the Dalmatian coach dog.

ON THE EXCRETION OF URIC ACID AND URATES BY THE BIRD

By N. B. DREYER and E. GORDON YOUNG

(From the Departments of Biochemistry and Pharmacology, Dalhousie University, Halifax, Canada)

Under luminal anesthesia the ureteral excretion of the cockerel is a viscous fluid containing solid matter which consists of uric acid or urate or a mixture of both. The pH range is 5.0 to 6.0, with an average of 5.4. The concentration of urate is usually at a

level of supersaturation, 0.2 to 0.7 per cent, and a variable portion of this urate has been demonstrated to be colloidal by ultrafiltration experiments. Under urethane anesthesia birds tend to excrete a clear urine, less acid and less concentrated than that under luminal.

Because of the condition of the urine a study has been made seeking to establish the effect of the intravenous injection of substances which might increase the solubility of the urate in the kidney mechanism. The urine has been collected after cannulation of both ureters. Colloidal preparations of monosodium urate and of hexamine urate are recovered between 40 and 100 per cent, while similar preparations of lithium urate are between 100 and 250 per cent. Concentrated piperidine urate is not recovered at all in 6 hours.

Of solvents for uric acid, lithium carbonate in isotonic concentration is most effective, inducing marked diuresis and increasing the output of uric acid manyfold. Lithium chloride is also very active. Piperidine, piperazine, and ethylene diamine are less effective. Methyl amine, guanidine, and hexamethylenetetramine are ineffective. Hypertonic solutions of glucose, sodium sulfate, sodium chloride, and sodium bicarbonate cause diuresis with increased output of urate.

Caffeine, theophylline, euphylline, colchicine, and adrenalin are all very effective in promoting diuresis and increased output of uric acid. Theobromine is entirely negative, as is the salicylate group of compounds.

THE TOTAL METABOLISM OF THE KING SNAKE, WITH SPECIAL REFERENCE TO THE SPECIFIC DYNAMIC ACTION OF FOOD

By R. A. KOCHER

(From the Grace Deere Velie Metabolic Clinic, Carmel-by-the-Sea, California)

No satisfactory information is available on the effect of feeding on the respiratory exchange of cold blooded animals. The present experiments were carried out on the king snake by the use of the modified Benedict rat unit apparatus with constant temperature bath. Determinations of the respiratory quotient during fasting and after voluntary and forced feeding were made with a complete

balance sheet of the intake and output during the entire period of digestion. Experiments were also carried out to determine the effect of various temperatures on the fasting metabolism and the specific dynamic action of foods. The respiratory exchange of the king snake was found to increase over 1000 per cent during the height of digestion, with a rise of body temperature of 2-3°. The metabolic rate was increased by external temperature but did not follow van't Hoff's law. A marked retention of nitrogen occurred during the period of digestion of protein.

QUANTITATIVE DETERMINATIONS OF URIC ACID IN GLOMERULAR URINE AND BLOOD PLASMA FROM FROGS AND SNAKES

By JAMES BORDLEY, 3RD, AND A. N. RICHARDS

(From the Laboratory of Pharmacology, University of Pennsylvania, Philadelphia)

Folin's method has been adapted to the estimation of amounts of uric acid of the order of 0.000005 mg. with accuracy comparable to that attained when conventional units are used. Analysis of glomerular fluids taken from the renal corpuscles of frogs and snakes shows the concentration of uric acid in these fluids to be of the same order as that of the blood plasmas from which these are derived.

REDUCING SUBSTANCES AND PHOSPHATES IN GLOMERULAR URINE, AQUEOUS HUMOR, CEREBROSPINAL FLUID, AND BLOOD PLASMA OF FROGS AND NECTURI

By ARTHUR M. WALKER, E. H. ELLINWOOD, AND JOHN A. REISINGER

(From the Laboratory of Pharmacology, University of Pennsylvania, Philadelphia)

The colorimetric methods of Sumner and of Kuttner have been adapted to the estimation of minute amounts of sugar and phosphate. The reducing power of glomerular urine from frogs appears to be about 5 per cent less than that of arterial blood plasma. The same is true of frogs poisoned with phlorhizin. The reducing power of cerebrospinal fluid and aqueous humor is about 30 per cent less than that of arterial blood plasma.

All of the inorganic phosphate of frog plasma is filtrable through

artificial membranes. The phosphate content of glomerular urine of frogs and *Necturi* and of the lymph of frogs is the same as that of the blood plasma. The phosphate content of aqueous humor and cerebrospinal fluid is approximately 50 per cent of that of the blood plasma.

THE MECHANISM OF LACTIC ACID OXIDATION BY THE α-HYDROXYOXIDASE OF GONOCOCCI

By E. S. GUZMAN BARRON and A. BAIRD HASTINGS

(*From the Lasker Foundation for Medical Research and the Department of Medicine, the University of Chicago, Chicago*)

A suspension of gonococci oxidizes α-hydroxy acids and α-ketonic acids with the formation of an acid of 1 carbon atom less and CO_2. When the bacterial suspension is kept in the ice box for 24 hours or more, there is left only the enzyme which oxidizes α-hydroxy acids, with α-ketonic acids as the final product of oxidation. In our experiments, lactic acid has been the α-hydroxy acid chosen as oxidizable substrate because the rate of its oxidation is greater than that of the other α-hydroxy acids tested. Lactic acid is oxidized to pyruvic acid. The enzyme *α-hydroxyoxidase* has been found to be made up of two components. One, which we may call the *activating coenzyme*, activates the substrate and renders it ready to be oxidized; the other, the *oxygen transfer enzyme*, performs the oxidation. These components of α-hydroxyoxidase can be separated by two methods: (*a*) by submitting the bacterial suspension to the action of increased temperatures; and (*b*) by the action of selective inhibiting substances. The oxygen transfer enzyme is more heat-labile than the activating coenzyme; the former is partially destroyed at 48° and completely destroyed at 52°; the latter is not destroyed until a temperature of 70° is reached. The former is inhibited by the action of selective inhibitors, such as KCN and valeronitrile, while the latter is entirely unaffected by them. When the oxygen transfer enzyme has been destroyed by the action of heat or has been inhibited by the action of selective inhibitors, the bacterial suspension is unable to oxidize lactic acid. In such a case this second component of α-hydroxyoxidase can be replaced with a reversible oxidation-reduction dye (*e.g.* cresyl blue), which, acting as a catalyst, readily oxidizes lac-

tic acid to pyruvic acid. Monoiodoacetate is an inhibitor of the oxygen transfer enzyme as well as of the artificial catalyst (reversible oxidation-reduction dye). The velocity of reaction between monoiodoacetate and the enzyme seems to be a slow process, for after the inhibition has reached its maximum limit it is possible to increase the rate of oxidation of lactate by the addition of new amounts of the artificial catalyst.

THE EFFECT OF pH ON THE ANAEROBIC METABOLISM OF ISOLATED FROG MUSCLE

By MARGARET KERLY AND ETHEL RONZONI

(*From the Laboratory of Biological Chemistry, Washington University School of Medicine, St. Louis*)

5 to 7 gm. of muscle were placed in Ringer's solution containing 0.02 M sodium bicarbonate and equilibrated with a mixture of nitrogen and carbon dioxide in such proportions as to give the required pH. Muscles from opposite legs were used as resting controls. The muscles were frozen in carbon dioxide snow and extracted with a solution of 2.5 per cent mercuric chloride in 0.5 N hydrochloric acid. Such a solution extracts some of the glycogen and all of the acid-soluble carbohydrates and phosphates present in the muscle. A portion of this extract and the solid residue were hydrolyzed separately, the hydrolysates treated with mercuric sulfate, and the fermentable reducing substances estimated by the Shaffer-Hartmann method. The sum of these two values gave the total carbohydrate content. The remainder of the acid extract we freed from mercury with hydrogen sulfide, which procedure removed all the glycogen present; reducing substances and hexosemonophosphate were estimated in the mercury-free filtrate.

At pH 9.0 the loss in total carbohydrate is approximately balanced by the gain in lactic acid, but at a neutral or acid reaction the lactic acid increase is not so great as the carbohydrate decrease. Increase in hexosemonophosphate only partially accounts for this discrepancy. At pH 6.0 the lactic acid does not increase for the first few hours; during this time the muscles produce basic substances, but after about 3 hours acid is formed. At this point the hexosemonophosphate content is at a maximum and other experiments show that all the phosphocreatine is broken down. If

anaerobiosis is continued for about 20 hours, there is a large forma-
tion, 300 to 400 mg. per 100 gm. of reducing substance, fermenting
at the same rate as glucose.   Even at neutral and alkaline reac-
tions there is some increase in fermentable reducing substance.
Precipitation of the mercuric chloride extract with alcohol gives
no evidence of the presence of any carbohydrate, other than glyco-
gen, insoluble in 66 per cent alcohol.   In resting muscles there are
only traces of any alcohol-soluble polysaccharide having an in-
creased fermentable reducing value on hydrolysis, but in muscles
kept at pH 6.0 for several hours there is such a compound present.

THE RÔLE OF METHEMOGLOBIN IN METHYLENE BLUE CATALY-
SIS OF LACTIC ACID OXIDATION

By WILLIAM B. WENDEL

(From the Laboratory of Biological Chemistry, Washington University
School of Medicine, St. Louis)

We have previously reported[25] that the influence of cyanide
on the rate of oxidation of lactic acid by dog erythrocytes in the
presence of methylene blue is not to inhibit but, rather, to acceler-
ate the oxidation.   This fact is of interest in relation to possible
interpretations of the mechanism of methylene blue catalysis.
Results of further experiments appear to indicate that methemo-
globin is not essential to lactate oxidation in this system.   This
is suggested by the following facts:

1. Concentrations of HCN which increase the rate of lactic acid
oxidation by cells containing methylene blue partially inhibit this
oxidation by cells containing methemoglobin but no methylene
blue; i.e., cells treated with amyl nitrite (Warburg, Kubowitz,
and Christian[26]).

2. Cells which have been previously incubated with methylene
blue and cyanide until all the hemoglobin has been converted to
cyanmethemoglobin oxidize in closed filled tubes only such an
amount of lactic acid as can be accounted for by the dissolved
oxygen and methylene blue.   The cyanmethemoglobin does not,
therefore, act as a lactate oxidant.   In the presence of oxygen,

[25] Wendel, W. B., J. Biol. Chem., **92**, p. xlvii (1931).

[26] Warburg, O., Kubowitz, F., and Christian, W., Biochem. Z., **227**, 245
(1930).

however, such cells rapidly oxidize lactic acid, indicating that methylene blue catalysis occurs when methemoglobin is inactive.

The following observations lead to the conclusion that the accelerating action of cyanide is not concerned with *any* phase of the oxygen-activating mechanism, and that it is probably due to the union of HCN with the product of the oxidation; namely, pyruvic acid.

1. The accelerating action of HCN is negligible when the concentration of methylene blue is less than that which is necessary to produce the maximal rate of lactic acid oxidation; *i.e.*, when the rate of oxygen activation and not the rate of substrate activation is the limiting factor.

2. Cyanide does not accelerate oxidation unless added in sufficient quantities to give an excess over and above that which is rapidly and firmly bound by the methemoglobin formed by the action of methylene blue on hemoglobin. With increasing concentrations of HCN above this minimal value (about 0.01 M), the acceleration progressively increases up to 0.06 M, above which concentration (up to 0.1 M) there is no further effect.

3. HCN combines with pyruvic acid, presumably to form the cyanhydrin, under the experimental conditions.

4. Semicarbazide, which also combines with pyruvic acid, accelerates the oxidation in a manner analogous to that of cyanide.

THE INFLUENCE OF MONOIODOACETATE ON OXIDATION AND FERMENTATION BY YEAST

By ELLEN EHRENFEST

(*From the Laboratory of Biological Chemistry, Washington University School of Medicine, St. Louis*)

The inhibitory effect of iodoacetate on yeast (bakers') fermentation and oxidation was studied by means of the Warburg technique.

Iodoacetate in a dilution of 1:56,000 completely inhibits fermentation at pH 4.6, if the iodoacetate is added a short time before the glucose. If added simultaneously with glucose, complete inhibition occurs only after 70 minutes.

Higher concentrations of iodoacetate are required to prevent fermentation when yeast is suspended in phthalate, citrate, or

acetate than when it is suspended in phosphate or unbuffered solutions.

The degree of inhibition of fermentation depends upon pH. If iodoacetate and glucose are added to yeast in a phosphate buffer (making the final concentration of iodoacetate 1:28,000), the inhibition is complete at pH 4.6, but decreases with increasing pH until there is no inhibition at an initial pH of 7.0. However, at the latter pH, higher concentrations of iodoacetate cause partial inhibition (80 per cent with 1:2000).

The varying effect of iodoacetate presumably is due to changes in cell permeability. Yeast, suspended for 2 hours in a phosphate buffer of pH 7.0 containing iodoacetate (1:28,000), ferments normally on subsequent addition of glucose; if during fermentation, however, sufficient acid is added to change the pH to 4.6, fermentation stops. On the contrary, if the suspension is washed and resuspended in glucose and phosphate (pH 7.0 and iodoacetate-free), it ferments at the normal rate even after addition of acid. This shows that the failure of inhibition is not due to the destruction of iodoacetate at pH 7.0. Indeed, in a solution of muscle enzymes the inhibitory effect of this poison has been demonstrated, at any pH between 6.5 and 9.0. The inactivity of zymin preparations at pH 7.0 or above renders them useless for demonstration of this point. The inability to restore the fermentative power of yeast that has been standing in iodoacetate at pH 4.6, even by repeated washing, is further evidence that the effect noted at pH of 7.0 is on the permeability of the yeast cell.

When fermentation of glucose is completely inhibited by iodoacetate, no oxidation of glucose occurs. Under these conditions, however, lactate, pyruvate, and alcohol are oxidized, though at a somewhat slower rate than in the absence of iodoacetate. If iodoacetate and glucose are added to the yeast suspension at the same time, fermentation is inhibited in a shorter time than oxidation. The accumulation of intermediates probably explains the lag between inhibition of fermentation and oxidation. These facts have certain interesting implications in regard to the separation of oxidation and fermentation of glucose into different processes, as claimed by Lundsgaard and others. Their observations that iodoacetate exerts little or no inhibition on yeast oxidation of glucose were probably due to the variety of yeast or to the con-

ditions of their experiments, the iodoacetate being added after the fermentation and oxidation were under way and hence after alcohol or other intermediates had accumulated.

Evidence for accumulation of intermediates is furnished by the observation that in the early stage of glucose utilization the oxygen consumed accounts for only 25 per cent of the glucose disappearing.

THE EFFECT OF CYANIDE ON THE RESPIRATION OF RAT LIVER AND KIDNEY

By EDWARD MUNTWYLER AND DOROTHY BINNS

(*From the Department of Biochemistry, School of Medicine, Western Reserve University, Cleveland*)

According to Warburg's theory of cell respiration, the oxidation agent of respiration is molecular oxygen and the primary reaction is between molecular oxygen and iron.[27]   This conclusion was the result of a study of a number of systems in which the addition of cyanide inhibited oxidation, while the addition of minute quantities of iron acted markedly as a catalyst in certain reactions. Dixon and Elliott[28] have recently made an extensive study of the effect of cyanide on the respiration of various animal tissues. These workers observed that the maximum inhibition varies in different tissues between 40 and 90 per cent, the average value being about 60 per cent.   From this it was concluded that Warburg's "respiratory enzyme" (Keilin's cytochrome-indophenol-oxidase system) can therefore only account at the most for about two-thirds of the total respiration of animal tissues.

A study was begun in this laboratory to compare certain tissue reactions (mainly on liver) of normal rats and rats made anemic by being placed solely on a milk diet.   It was observed that the indophenol-oxidase color reaction with the "Nadi" reagent was markedly less intense in the liver of severely anemic animals than in the liver of normal animals.   Since cyanide completely inhibits the indophenol color reaction, it seemed interesting to compare the effect of cyanide on the respiration of liver tissue with the degree of indophenol-oxidase color reaction.

[27] Warburg, O., *Biochem. Z.*, **119**, 134 (1921); **142**, 518 (1923).
[28] Dixon, M., and Elliott, K. A. C., *Biochem. J.*, **23**, 812 (1929).

The respiration studies were made usually on tissue slices suspended in the Ringer's solution described by Richardson.[29] A comparison was also made of the effect of cyanide on the respiration of liver tissue suspended in Ringer's solution containing no phosphate and in Ringer's solution containing an M/15 phosphate concentration. The cyanide concentration usually employed was M/150.

The rate of oxygen uptake of liver tissue of normal animals, as well as of anemic animals treated for varying periods with iron or iron and copper, is relatively constant. Kidney tissue obtained from normal and anemic animals shows the same rate of oxygen uptake although it is at a greater rate than liver tissue.

The inhibition of the respiration of liver tissue by cyanide over a 2 hour period of observation varied from 31.5 to 72.1 per cent. The degree of inhibition was in no relation to the degree of indophenol-oxidase color production.

The inhibition of the respiration of liver tissue by cyanide in the Ringer's solution containing no phosphate and in Ringer's solution containing an M/15 phosphate concentration showed no essential difference.

OPTICALLY ACTIVE 5,5'-PHENYLETHYLHYDANTOINS

By HARRY SOBOTKA, S. M. PECK, AND JOS KAHN

(From the Laboratories of the Mount Sinai Hospital, New York)

The authors were able to separate 5,5'-phenylethylhydantoin into its optically active components. Several derivatives were studied and a synthesis of the optical antipodes starting from dl-phenylethylglycine was accomplished.

The dl-phenylethylhydantoin has been in use as a hypnotic since 1916 and causes toxic skin manifestations and other symptoms commonly designated "nirvanol disease." The authors, in cooperation with Dr. Bela Schick, could show that the toxicity is mostly due to the levorotatory form, while the hypnotic effect of the much less toxic dextro form is at least half that of the levo form. To explain the influence of optical activity on nirvanol disease, the hypothesis is offered that preferably the levorotatory

[29] Richardson, H. B., Physiol. Rev., 9, 61 (1929).

form of phenylethylhydantoin or its nearest derivatives com-
bines with serum protein as a "spontaneous haptene" causing
symptoms of allergic nature.

THE MECHANISM OF SACCHARINIC ACID FORMATION

By BEN H. NICOLET

(*From the Research Laboratories of the Bureau of Dairy Industry, United
States Department of Agriculture, Beltsville, Maryland*)

α-Hydroxy-β-methoxy-β-phenylpropiophenone, $C_6H_5CH(OCH_3)$-
$CH(OH)COC_6H_5$, which may be considered as a monomethylated
diphenyl triose, reacts smoothly with alkali to form diphenylactic
acid, $C_6H_5CH_2CC_6H_5(OH)CO_2H$.   This is the characteristic re-
action of saccharinic acid formation, but with the loss of methyl
alcohol instead of water.   Since the hydrogen of the β-hydroxyl
group is here replaced by methyl, there is here no room for doubt
that it is the β-carbon atom from which oxygen separates, under
the influence of alkali.   This corresponds to Nef's first theory of
the reaction in question, but differs from his final theory.   The
first stage of the reaction involved in the transformation of a
sugar to a saccharinic acid is thus analogous to the dehydration
of an aldol to give an unsaturated aldehyde.   It is not unlikely
that the splitting of the carbon chain in sugars by alkalies is also
an aldol reaction—a reversal of the aldol condensation—instead
of a methylene dissociation as claimed by Nef.

THE TRANSFORMATION OF d-GLUCOSE INTO d-FRUCTOSE BY PYRIDINE

By W. C. AUSTIN AND WALTER PRUSAIT

(*From the Department of Physiological Chemistry, Loyola University School
of Medicine, Chicago*)

Studies of the reaction of isomerization of d-glucose in pyridine
to form d-fructose, first reported by Danilov[30] and coworkers,
have been made.   Samples of d-glucose have been heated in pyri-
dine, the purity of the pyridine, the ratio of the pyridine to the
glucose, the temperature and pressure, and the duration of the
heating being varied.   After the removal of the unchanged pyridine

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[30] Danilov, S., Venus-Danilova, E., and Schantarovich, P., *Ber. chem.
Ges.*, **63**, 2269 (1930).

the reaction products have been tested for specific rotation, reducing hexose, and aldohexose. The results obtained indicate that from 1 to 10 per cent of the d-glucose may be transformed into d-fructose, whereas Danilov reported values of 20 and 38 per cent in two experiments. From 10 to 50 per cent of the sugars is destroyed by side reactions during the heating. Further experiments are in process with the object of increasing the amount of isomerization and further limiting the destruction of the sugars.

THE NITROGEN OF ALKALINE MERCURY FILTRATES

By MARK R. EVERETT and FAY SHEPPARD

(From the Department of Biochemistry and Pharmacology, University of Oklahoma Medical School, Oklahoma City)

Contrary to general belief[31] mercuric salts and alkali do not completely precipitate the nitrogenous substances from biological fluids. We have made a comprehensive study of this precipitation and find that the following substances will appear in the filtrate: the acylated amino acids (hippuric acid, etc.), anilides, the amides of several monobasic acids, alkylamines, methyl purines (caffeine and theophylline), methylamino acids (sarcosine), betaines, choline, a large variety of aliphatic and dibasic amino acids (but not leucine, glycine, cysteine, tyrosine, tryptophane, or the hexone bases), certain heterocyclic compounds (proline, succinimide, oxyproline, piperidine, pyridine, piperazine, and the methylated nitrogen ring compounds), and salts of thiocyanic and nitric acids. The relation of methyl and acyl groups in nitrogen compounds permits the prediction of their behavior. The previously unknown mercuric salts of some of these compounds have been prepared by one of us.

In muscle extracts about one-fifth of the nitrogen is in the mercuric filtrate. In urine this fraction is much smaller quantitatively but is nevertheless quite definite and comprises some of the substances enumerated above, as proved by their isolation. Hence the claim that the sugar of normal urine cannot be nitrogenous is not proved. Hippuric acid and its homologues lead to erroneous results with the osazone reaction in such filtrates. Certain limi-

[31] Freund, E., and Fellner, R., *Z. physiol. Chem.*, **36**, 401 (1902). Benedict, S. R., and Osterberg, E., *J. Biol. Chem.*, **34**, 195 (1918). Lustig, B.,

tations of the Kjeldahl method appear with this fraction of nitrogen.

NATURE OF THE FERMENTABLE SUGAR IN NORMAL URINE

By EDWARD S. WEST, R. C. NORRIS, AND ALEXANDER STEINER

(From the Laboratory of Biological Chemistry, Washington University School of Medicine, St. Louis)

Large quantities of $HgSO_4$-$BaCO_3$ filtrates of urine were prepared according to the procedure of West and Peterson. These were evaporated to dryness *in vacuo* at a low temperature, the residue taken up in a little water, and precipitated with a large volume of alcohol. The alcohol solutions were evaporated to dryness *in vacuo*, taken up in 0.15 M NaH_2PO_4, and fermented in a Warburg apparatus. Control fermentations of various sugars were run simultaneously. The results point to the conclusion that glucose is the fermentable sugar of both normal urine and urine in fasting.

MORE COMPLETE OXIDATION OF SUGARS IN ALKALINE SOLUTION IN PRESENCE OF CYANIDE

By BEN K. HARNED AND CHARLES J. DEERE

(From the Department of Chemistry, University of Tennessee School of Biological Sciences, Memphis)

Cyanides have been employed extensively in biological oxidation studies to inhibit those oxidations in which the heavy metals act as catalyst, with little or no regard for other effects simultaneously produced. The addition of cyanides to tissue suspensions and living organisms usually results in a 40 to 90 per cent inhibition in oxygen consumption. However, in some cases no inhibition occurs and even acceleration has been recorded.

With the view that a quantitative study of the action of cyanide on a relatively simple organic system might elucidate some of the effects observed in tissues, we have studied the effect of cyanide on the oxidation of reducing sugars by molecular oxygen. We have been able to show that under optimum conditions cyanide increases the oxygen consumption of alkaline glucose solutions 60 per cent, with only a slight increase in the *velocity* of the reaction. The formic acid production is the same as in the absence of cyanide, but CO_2 production is increased. The close agreement be-

tween the velocity constants, with and without cyanide, calculated from oxygen absorption values, is interpreted as indicating that catalysis by heavy metals is negligible in the oxidation of alkaline sugar solutions by molecular oxygen in our experiments.

There is always a small loss in cyanide which may have been oxidized through the peroxidation of the sugars.[32]   This behavior may be related to the detoxication of cyanide in the living organism.

GALACTOSE UTILIZATION FOLLOWING COMPLETE REMOVAL OF THE LIVER

By MARSCHELLE H. POWER

(From the Divisions of Chemistry and of Experimental Surgery and Pathology, The Mayo Foundation, Rochester, Minnesota)

If galactose is injected intravenously in normal dogs (500 mg. for each kilo of body weight) it disappears from the blood in 2 hours, and within the same time 20 to 25 per cent of the amount given appears in the urine.   In similar experiments carried out in conjunction with Drs. Bollman and Mann on hepatectomized dogs there occurs a similar disappearance of galactose from the blood, but 50 to 60 per cent is recovered in the urine.   There is greater delay in the clearance of galactose from the blood of nephrectomized animals and a still greater delay when the liver is also removed.   Administration of galactose appears to be without effect on the hypoglycemia of hepatectomized animals.   Evidence could not be obtained of the conversion of galactose to glucose by animals without a liver, although the conversion of fructose to glucose may be readily demonstrated under similar conditions.

THE EFFECT OF GLUCOSE INGESTION ON THE UREA, TOTAL NON-PROTEIN NITROGEN, AND CHLORIDE CONCENTRATION IN THE BLOOD

By HERMAN O. MOSENTHAL AND MAURICE BRUGER

(From the Department of Medicine, New York Post-Graduate Medical School and Hospital, New York)

60 glucose tolerance tests were carried out on 54 unselected patients.   The variations in the sugar, urea nitrogen, total non-pro-

tein nitrogen, and chloride concentrations in the blood were followed in most cases at intervals of 20 minutes, 40 minutes, 1 hour, 2 hours, and 3 hours following the ingestion of 100 gm. of glucose. The excretion of sugar in the urine and the urine volume output were also studied. The blood sugar was determined in every instance and the patient's tolerance for carbohydrate interpreted. The variations in the urea nitrogen concentration in the blood were followed in forty-five cases, the total non-protein nitrogen in thirteen, the whole blood chlorides in sixteen, and the plasma chlorides in six.

In 65 per cent of the cases, there was a gradual and appreciable fall in the urea nitrogen and total non-protein nitrogen concentrations in the blood following the glucose ingestion. In subjects with normal carbohydrate tolerance, there was no apparent reciprocal relationship between the decrease in these constituents and the rise in blood sugar, since the urea nitrogen and non-protein nitrogen showed a continuous diminution even after the blood sugar had returned to normal. The decrease of these substances in the blood was somewhat more marked in those cases with diminished sugar tolerance than in those with normal carbohydrate tolerance. The diminution in the urea nitrogen concentration in the blood was greater than that of the total non-protein nitrogen, with the result that the ratio of urea nitrogen to the total non-protein nitrogen fell decidedly in the majority of cases. In the remaining instances, the urea nitrogen and the total non-protein nitrogen either remained unchanged or showed a slight increase in their respective concentrations in the blood. There seemed to be no definite relationship between the variation of these substances in the blood and the degree of the diuretic response.

In nine cases the chloride concentration of the blood decreased following the glucose ingestion. In many instances there was a definite reciprocal relationship between the blood sugar and the blood chlorides. In cases with normal sugar tolerance, the return of the blood sugar to the normal value within 2 hours was associated with a similar return of the blood chlorides to the control level. In patients with diminished carbohydrate tolerance, the elevated blood sugar at the end of 2 or 3 hours was accompanied by a persistent diminution in the blood chlorides. In seven other studies, the blood chlorides remained practically unchanged and only insignificant increases were occasionally noted. Simultaneous

determinations of whole blood and plasma chlorides in six cases showed that identical variations occurred in the plasma chloride concentration following glucose ingestion.

PLASMA PHOSPHATES DURING CHANGES IN CARBOHYDRATE METABOLISM IN NORMAL AND ADRENALECTOMIZED ANIMALS

By CARL F. CORI AND GERTY T. CORI

(*From the Department of Pharmacology, Washington University School of Medicine, St. Louis*)

When normal rabbits received an intravenous injection of a non-convulsive dose of crystalline insulin,[33] the inorganic phosphates in plasma were noticeably diminished after 20 minutes, reached their lowest point after 45 minutes, and then slowly returned to the original level. In adrenalectomized rabbits insulin injections produced practically no change in plasma phosphates, in spite of the fact that the blood sugar fell to very low levels. Convulsions in both normal and adrenalectomized animals caused a marked rise in plasma phosphates. Coincident with the decrease in plasma phosphates, insulin produced an increase in the blood lactic acid of normal rabbits, while in adrenalectomized rabbits blood lactic acid did not change. Epinephrine injections lead to a decrease in plasma phosphates and increase in blood lactic acid. The changes in phosphate and lactic acid produced in normal animals by insulin injection are ascribed to a reflex discharge of epinephrine elicited by the hypoglycemia, the epinephrine causing an accumulation of hexosemonophosphate in muscle, accompanied by a withdrawal of inorganic phosphate from the blood and liberation of lactic acid into the blood.

Glucose injected intravenously in fasted rabbits did not produce significant alteration in the plasma phosphate level within 2 hours. Injection of glucose plus insulin was followed by a prompt fall in plasma phosphates, not only in normal but also in adrenalectomized animals. When the relation of injected glucose to insulin was such that hypoglycemia developed after 1 to 1½ hours, the plasma phosphates continued to fall in the intact animal due to the secondary output of epinephrine, while they returned to the original level in the adrenalectomized animal, resulting in a

[33] The authors are indebted to Dr. E. M. K. Geiling for the crystalline insulin.

crossing of the sugar and phosphate curves. The immediate fall in plasma phosphates after glucose plus insulin injection seems to be related to the rapidity of sugar utilization, because glucose alone did not produce it and because the inorganic phosphates reappeared in the adrenalectomized animal when most of the injected sugar had been used up.

THE ESTIMATION OF BLOOD DIASTASE

By MICHAEL SOMOGYI

(From the Laboratory of the Jewish Hospital of St. Louis, St. Louis)

All the existing methods have the basic defect in common that they employ soluble starch as a substrate in measuring diastatic activity. It is practically impossible to procure two identical soluble starches even if prepared by one definite process and by one and the same manufacturer. As a consequence, various starches react differently with identical quantities of enzyme, under otherwise identical conditions, rendering it impossible to correlate the findings of various investigators. Besides this, other serious sources of error are encountered. In the numerous procedures based upon the amyloclastic action of diastase, difficulty enters at the determination of the point where the starch-iodine reaction terminates. The color scale produced with iodine by the array of cleavage products allows no semblance of objective discernment. In the other group of methods, based upon the quantitative determination of reducing sugars formed from starch, peculiarities of the kinetics of the enzyme reaction have been wholly disregarded, and as a result increasing quantities of enzyme do not yield proportional amounts of reducing sugars.

The error due to the variability of substrate was obviated by the use of starch pastes prepared from refined raw starches. A number of different starches were compared and found to yield identical results. Soluble starches give higher results, while glycogen behaves entirely like pastes of raw starches.

We have evolved two procedures. In measuring the amyloclastic activity, the difficulty of identifying colors was mitigated by the introduction of transparency as an additional criterion. The amounts of starch, of blood serum, and of iodine are so standardized that enzyme action—between certain limits—is in quantitative relation to the time needed to break down the starch to a

stage defined by color plus transparency after the addition of iodine.

For the method in which the reducing sugars formed by the enzyme are determined, we have established the limits of enzyme to substrate ratios within which the quantity of sugar produced is in direct proportion to that of enzyme. This method is far finer than the first.

It is of interest that the results obtained by the two procedures run parallel. This fact is in favor of the view that the initial (amyloclastic) phase in the cleavage of starch and the final one (sugar formation) are attributable to a single enzyme.

FURTHER STUDIES ON ANTIUREASE

By J. STANLEY KIRK and JAMES B. SUMNER

(From the Department of Physiology and Biochemistry, Cornell University Medical College, Ithaca)

Antiurease is produced by immunizing rabbits or chickens with crystalline urease.[34] It precipitates urease at high dilution and at the same time inhibits the action of urease upon urea. In earlier work we determined antiurease activity by measuring the decrease of urease activity after incubating urease with antiurease. However, the precipitate of urease-antiurease possesses a high urease action and it appears best to centrifuge this precipitate off and to determine the amount of unprecipitated urease in the supernatant liquid. Hence our present method of expressing antiurease activity is a measure of the ability of antiurease to precipitate urease.

As already described[35] antiurease can be purified by adding crystalline urease to immune rabbit serum and incubating at 37°, centrifuging, washing the precipitate several times with physiological saline, decomposing with dilute hydrochloric acid, neutralizing with alkali, and centrifuging down the denatured urease. From 70 to 95 per cent of the antiurease originally present is recovered in the supernatant liquid. The amount of purification is considerable and, of course, varies, depending upon the concentration of antiurease started with.

[34] Kirk, J. S., and Sumner, J. B., J. Biol. Chem., 94, 21 (1931). Howell, S. F., Proc. Soc. Exp. Biol. and Med., 29, 759 (1932).

[35] Sumner, J. B., and Kirk, J. S., Z. physiol. Chem., 205, 219 (1932).

Purified antiurease is probably a globulin. It is precipitated by dialysis and the precipitate redissolves in salt. It is destroyed by long dialysis. While stable at 30° at pH 5.0 it is rapidly destroyed by unactivated papain. However, at pH 7.0 neither unactivated papain nor activated trypsin destroys antiurease. Preliminary experiments made by estimating the antiurease activity of purified antiurease solutions and later determining the dry weight of salt-free material gave 80 anti units per mg. (two experiments). Experiments based on weighing the precipitate of urease-antiurease and then subtracting the calculated amount of urease present (133 urease units per mg.) gave 85 anti units per mg. of antiurease (three experiments).

HOMOGENTISIC ACID: A PHYSIOLOGICAL OXIDATION-REDUCTION SYSTEM

By ELLA H. FISHBERG and B. T. DOLIN

(From the Biochemical Laboratory of the Beth Israel Hospital, New York)

A deep pigmentation of the cartilage, ligaments, and periosteum is the essential characteristic of ochronosis. It is often found associated with a specific familial metabolic anomaly, alkaptonuria. The urine of these patients reduces Benedict's solution, due to the presence of homogentisic acid, chemically 2,5-dihydroxyphenylacetic acid. It is also found as a result of the continued application of carbolic acid to open wounds over a long period of time. The circulation of these hydroxylated aromatic compounds in the blood results in the deposition of the specific pigment in the localizations mentioned above. The chemical structure of homogentisic acid, with its intimate relation to the quinones, marks it as a possible physiological oxidation-reduction system in the body.

It was found on oxidative titration with bright platinum electrodes in strongly buffered solutions to give very steady and easily reproducible potentials. It is slightly more negative than the quinone-hydroquinone system, due to the side chain substitution. Its rH was 21.7.

The quinone double bonds in the benzene ring in the para position make the compound strongly chromogenic. There is a great tendency to the formation of meriquinones, compounds consisting of an oxidized and a reduced half. These are all intensely colored compounds. Sections of cartilage, when allowed to stand in

tion. In acid solution they pass through an intermediate state of intense red color. In how far these intermediate stages can be correlated with the intermediate stages in the oxidation of the homogentisic acid quinoid group remains for further study.

THE QUANTITATIVE ESTIMATION OF POTASSIUM IN SMALL AMOUNTS OF SERUM, WITH A STUDY OF THE COBALT-CYSTEINE-HYDROGEN PEROXIDE COMPLEX

By ALBERT E. SOBEL AND BENJAMIN KRAMER

(From the Pediatric Research Laboratory, The Jewish Hospital of Brooklyn, Brooklyn, New York)

This method depends upon the colorimetric determination of cobalt as the cobalt-cysteine-hydrogen peroxide complex.

To 0.2 cc. of serum, in a specially designed centrifuge tube, 0.4 cc. of the Kramer-Tisdall cobalti-nitrite reagent is added. This is allowed to stand 45 minutes and is washed three times with 30 per cent alcohol. The precipitate is dissolved in 6 N HCl and evaporated to dryness. The color is developed and compared against a cobalt standard. The molar ratio of potassium to cobalt in the precipitate is 2:1.17. It is very constant under the conditions given.

In an alkaline phosphate solution cobalt forms an olive-green complex with cysteine. On addition of at least 0.5 mol of H_2O_2 for each mol of cysteine an intense bright yellow complex forms. When the molar ratio of cysteine-0.5 H_2O_2 to cobalt is at least 24:1, the intensity of the color is directly proportional to the amount of cobalt present.

In various normal and pathological cases examined the potassium concentration of serum varies from 12.4 to 26.5 mg. per cent.

THE DETERMINATION OF NITRATE NITROGEN IN PLANT TISSUES

By HUBERT BRADFORD VICKERY, GEORGE W. PUCHER, AND ALFRED J. WAKEMAN

(From the Biochemical Laboratory of the Connecticut Agricultural Experiment Station, New Haven)

In the course of experimental work upon the development of a method to determine the quantity of organic acids in plant tissue

it was found that nitric acid can be quantitatively extracted by ether under certain conditions.   A 2 gm. sample of the powdered air-dry tissue is mixed with sufficient 4 N sulfuric acid to bring it to a reaction of pH 1.0; the quantity required is determined for each specimen in advance and is usually found to be 3 to 4 cc. 3.5 gm. of asbestos are mixed with the acidified sample and the material, after being transferred to a paper extraction thimble, is extracted with alcohol-free ether for a period of at least 8 hours. The extract is treated with sufficient 0.5 N sodium hydroxide to make it alkaline to phenolphthalein, the ether is evaporated, and the aqueous residue is made to 100 cc.   The nitrate in a suitable aliquot part of this solution is reduced with acid and iron powder and the ammonia so produced is distilled and determined by the Nessler method.

It was found that potassium nitrate, added to asbestos in quantities equivalent to from 1 to 20 mg. of nitrogen, could be recovered with an average precision of 98.8 per cent; and nitrate added to samples of tobacco leaf of known nitrate content was practically quantitatively recovered.

THE DETERMINATION OF IODINE IN BLOOD, TISSUES, AND FOOD

By EMIL J. BAUMANN AND NANNETTE METZGER

(From the Laboratory Division, Montefiore Hospital, New York)

To avoid the losses that are almost inevitable in the micro determination of iodine in blood, tissues, etc., a procedure has been developed in which the organic matter is destroyed by burning in a stream of oxygen in closed vessels.   The resulting smoke cloud, which contains 25 to 50 per cent of the iodine present, is passed through a gas wash bottle containing water and a little alkali, then through two funnels placed mouth to mouth with several filter papers between them, and finally through another wash bottle.   The funnels are held in a clamp and made gas-tight by coating the edges with paraffin.   This simple apparatus effectively absorbs the smoke cloud.

All the parts are washed off into a beaker in which the filter papers are also placed.   The washings are warmed, filtered, and evaporated to dryness, finally in a small platinum dish, the reaction being kept alkaline with caustic.   The dish is then heated with a small heating unit in a flask to remove the small amount of

organic matter still present. The iodine is then estimated by methods similar to those of Leitch and Henderson and of Kelly and Husband.

When 0.001 mg. of iodine is present, the error of the method is about 10 per cent; when 0.002 to 0.01 mg. is present the error is 5 per cent.

THE NATURE OF THE "ACID-SOLUBLE" PHOSPHORUS IN PLASMA

By ISIDOR GREENWALD AND IRVING LEVY

(*From the Department of Chemistry, New York University and Bellevue Hospital Medical College, New York*)

Chiefly because all, or nearly all, of the "acid-soluble" phosphorus of plasma, or serum, reacts with molybdic acid in the cold to form compounds that are reduced by stannous chloride or by other reducing agents, it is generally assumed to be inorganic phosphate. However, the present investigation has shown that a number of organic phosphoric acids, including practically all of the "acid-soluble" phosphorus of *whole blood*, react with molybdic acid to form complexes that are precipitated by protein. This finding raises the question as to whether the term "inorganic phosphate" can properly be applied to the compound or compounds that react with molybdic acid to form reducible complexes. Further evidence would appear desirable.

COMPARISON OF RESULTS OBTAINED BY THE BENEDICT OXY-CALORIMETER WITH THOSE BY ELEMENTARY ANALYSIS

By MILDRED ADAMS, JESSE L. BOLLMAN, AND WALTER M. BOOTHBY

(*From the Metabolism Laboratory, The Mayo Clinic, Rochester, Minnesota*)

A Benedict oxycalorimeter was attached to a Benedict Universal respiration table. Samples of food were burned in this, and both the carbon dioxide formed and the oxygen utilized were determined, and the non-protein respiratory quotient calculated, N being determined by the Kjeldahl method. These results will be compared with data obtained on the same samples by the usual methods of elementary analysis. Both sets of data were utilized to calculate the protein, carbohydrate, and fat content of the food for comparison with each other and with the fat as determined by

ether extraction. The calorific values of these were calculated, and in turn compared to the heat of combustion found by the bomb calorimeter; the carbohydrate as determined by weight difference was also compared with that calculated above.

THE TITRATION CONSTANTS OF AMINO ACIDS IN THE FORMOL TITRATION

By MILTON LEVY

(*From the Department of Chemistry, New York University and Bellevue Hospital Medical College, New York*)

Although the formol titration has been thoroughly studied from the view-point of stoichiometry, it is only recently that the attempt was made by Harris[36] to determine the titration constants of the amino acids in formaldehyde. Harris' conclusion that the dissociation constants are increased 1000-fold is valuable, but does not give an adequate description of the behavior of the amino acids.

Encouraged by Harris' statement that the hydrogen electrode behaves well in formaldehyde solutions, we have applied it to the titration of a number of amino acids with NaOH in formaldehyde. In 0.01 M solution of the amino acid the alkaline titration curves fit the Henderson-Hasselbalch equation and the titration constant varies with the formaldehyde concentration.

In order to follow the variation of the constant (pK_f) the pH values of solutions of the amino acids brought to the mid-point of the alkaline titration curves by the addition of NaOH were measured during the addition of formaldehyde. Plotting the pH of the solution against the logarithm of the formaldehyde concentration produced a series of curves all of which reach or approach a slope of 2 as the formaldehyde concentration increases. Extrapolation of this part of the curve to log $[CH_2O] = 0$ ($[CH_2O] = 3$ per cent) gives a constant (pK_{f_0}) which is useful in describing the behavior of the amino acids in the formol titration by means of the following equation.

$$pK_f = pK_{f_0} - 2 \log [CH_2O]$$

pK_f is the titration constant for the Henderson-Hasselbalch equation in formaldehyde of the concentration $[CH_2O]$. A more

[36] Harris, L. J., *Proc. Roy. Soc. London, Series B,* **104,** 412 (1930).

complete equation may be developed to cover the complete range of formaldehyde concentrations studied but is not of practical significance to the user of the formol titration. This equation, which will be published later, reduces to the one presented above at great formaldehyde and constant low amino acid concentrations. It may be observed that the equilibrium ordinarily assumed to exist in the formol titration does not lead to the above equation.

TABLE I

Formol Titration Constants of Amino Acids at 30°

Amino acid	pK'	pK$_{fo}$	End-point in 10 per cent CH$_2$O
			pH
Glycine..................................	9.58	6.62	7.6
Leucine..................................	9.50	7.90	8.9
Phenylalanine...........................	8.99	7.55	8.5
Tyrosine (0.004 M)......................	9.07	8.62	9.6
α-Amino-β-phenylacetic acid.............	8.84	6.85	7.9
Glutamic acid...........................	9.32	7.79	8.8

The theoretical end-points in the formol titration of the amino acids (99 per cent neutralized) in 10 per cent formaldehyde have been calculated from the constants to show the individual behavior and are given in Table I along with the values of pK$_{fo}$ and the titration constants in water. These figures are valid for 0.01 M amino acid and formaldehyde concentrations as used in the formol titration (5 to 15 per cent) and at 30°.

A RAPID MICRO METHOD OF CHLORIDE ANALYSIS

By LAWRENCE T. FAIRHALL and J. W. HEIM

(*From the Department of Physiology, School of Public Health, Harvard University, Boston*)

The determination of chlorides in body fluids may be made expeditiously and accurately by the following method even though the amount of material available is very small (0.05 to 0.10 cc.). In this modification of the iodometric method the protein constituent, the silver chloride, and the silver iodide are consecutively thrown down by centrifugation and the titration completed in the tube without transferring the contents at any point. To 0.5 cc. of water in a 15 cc. centrifuge tube introduce 0.1 cc. of the sample (serum, plasma, lymph, etc.). Wash down with 0.5 cc. of water,

add 1 cc. of tungstic acid protein precipitant, and centrifuge out the protein.   Add 1 cc. of standard silver nitrate solution acidified with HNO_3 and again centrifuge.   Add standard KI until near the end-point; then add the requisite amount of buffer and starch paste and centrifuge to remove the bulk of AgI, which otherwise obscures the end-point, and complete the titration.   In measuring the biological fluid and the silver nitrate solution great accuracy may be obtained by the use of pipettes made from thermometer tubing.

When this method is used with solutions of pure KCl varying in amount from 0.3 to 0.7 mg. for a given determination, the average error was 1 per cent in twenty-four determinations and the maximum error 2.7 per cent.   In serum and in serum containing known amounts of added KCl as well as in whole blood, the average error of thirty-one determinations was 2 per cent.   The determination may be entirely carried out in a single 15 cc. centrifuge tube and requires but 15 to 20 minutes for completion.

HEMOLYTIC ACTION OF SILVER OCCURRING AS AN IMPURITY IN CHEMICALLY PURE SODIUM CHLORIDE

By ERIC G. BALL

(*From the Laboratory of Physiological Chemistry, the Johns Hopkins University, School of Medicine, Baltimore*)

The erythrocytes of certain species of fish undergo rapid hemolysis when placed in isotonic solutions of certain brands of so called c.p. sodium chloride.   This hemolysis is now shown to be caused by an inorganic impurity in the sodium chloride which has been identified by means of the spectrograph as silver.

Isotonic solutions of silver-free sodium chloride are non-hemolytic.   Addition of silver nitrate to such solutions causes hemolysis similar in all respects to that produced by a solution of toxic salt, the rates of hemolysis being similar when the silver nitrate concentration is about 1×10^{-6} M.   On the basis of such a biological assay the toxic sodium chloride is calculated to contain silver chloride of the order of magnitude of 10^{-4} to 10^{-5} per cent.   Solutions of toxic sodium chloride may be purified by shaking with activated charcoal, the silver being adsorbed by such treatment.

Mammalian erythrocytes are more resistant to the hemolytic action of silver, the effect only being noticeable after prolonged

contact with the toxic solution. It is possible that some of the reported toxic effects of "pure" sodium chloride may be due to small amounts of impurities such as silver.

THE DISTRIBUTION OF CARBON DIOXIDE BETWEEN SOLUTIONS OF SODIUM BICARBONATE AND COTTONSEED OIL

By BYRON M. HENDRIX AND BERNARD BERNARDONI

(*From the Laboratory of Biological Chemistry, School of Medicine, University of Texas, Galveston*)

The observation of Jacobs that carbon dioxide diffuses rapidly through layers of fatty oils has made it seem of interest to determine the distribution of carbon dioxide between an aqueous solution of sodium bicarbonate and an oil. We have used a 0.05 M sodium bicarbonate solution which contained varying amounts of free carbon dioxide. Cottonseed oil was used for the oil phase. Two methods have been used in studying the distribution of the carbon dioxide in the two phases. The first one was an indirect method. The change in pH of the aqueous phase was followed. The increase in the pH of the bicarbonate solution was regarded as evidence that carbon dioxide had passed out of the solution into the oil. Adequate controls showed that carbon dioxide could not have been lost from the system. The second method was a more direct one in that the carbon dioxide in both the aqueous and oil phases was determined by the manometric method of Van Slyke and Neill.

The amount of carbon dioxide which passed into the oil varied inversely with the initial pH of the bicarbonate solution. This variation may be shown graphically by plotting the shift in pH and the carbon dioxide taken up by the oil against the initial pH. The condition at equilibrium is shown better, however, by using the pH of the solution at equilibrium instead of the initial pH. The curves plotted in any of the above ways are not straight lines. The amount of carbon dioxide taken up by the oil is relatively much greater in those experiments in which the initial pH was lowest; for example, when the aqueous phase had an initial pH of 6.70, 10.3 cc. of carbon dioxide were found in the oil phase when equilibrium was reached, while only 1.2 cc. of carbon dioxide were taken up by the oil when the initial pH of the solution was 8.2.

A SIMPLE PROCEDURE FOR THE DETECTION OF CARBON MONOXIDE IN BLOOD

By A. A. CHRISTMAN

(From the Laboratory of Physiological Chemistry, Medical School, University of Michigan, Ann Arbor)

The oxalated blood is allowed to react with potassium ferricyanide under diminished pressure in the gas chamber of the apparatus used by Van Slyke for the determination of the carbon dioxide capacity of blood. The gas mixture which results is passed into a special Hempel pipette containing a solution of palladium chloride. The presence of carbon monoxide in the blood results in the reduction of the palladium chloride to metallic palladium. Phosphomolybdic acid added to the solution containing metallic palladium is reduced, with the production of a colored solution varying from a light green to a deep blue, depending upon the amount of the metallic palladium present. The presence of carbon monoxide in the blood to the extent of 1 per cent may be detected by this procedure.

ON THE ESTIMATION OF UREA IN BLOOD AND URINE

By WALTER R. CAMPBELL

(From the Department of Medicine, University of Toronto, Toronto, Canada)

Substances soluble in the liquid may be heated to approximately 200° by adding ethylene glycol to their aqueous solution and heating over a free flame or in a glycerol bath. In this manner pure solutions of urea may be quantitatively hydrolyzed by adding to the urea solution in a 100 cc. pear-shaped tube 1.0 cc. of 10 per cent phosphoric acid and 2.0 cc. of ethylene glycol, boiling off the water, and raising the temperature to 190° for 20 minutes. After cooling and diluting, comparison is made with a standard solution of ammonium sulfate by Nesslerization, 15.0 cc. of Nessler's solution per 100 cc. being used.

After removal of preformed ammonia by means of permutit, urine is diluted 10 to 20 times, according as the specific gravity is below or above 1.018. An amount of the diluted urine to yield about 0.5 mg. of urea nitrogen (usually 1.0 cc.) is treated as above. Other normal urinary constituents do not interfere with the reaction. Added urea can be quantitatively recovered.

After precipitation of the blood proteins by zinc hydroxide the blood filtrate can be treated similarly. With very low blood urea values it is desirable to add known quantities of ammonium sulfate before Nesslerizing.

THE MECHANISM OF PARATHYROID HORMONE ACTION

By D. L. THOMSON and L. I. PUGSLEY

(From the Department of Biochemistry, McGill University, Montreal, Canada)

The increase in serum calcium which follows the intravenous injection of a potent parathyroid extract in dogs is not secondary to any fall in serum inorganic phosphate or $PO_4^\equiv$ ion, and may indeed be accompanied by a rise in phosphate concentration. Great decrease of the inorganic phosphate and $PO_4^\equiv$ ion concentration of the serum, following the administration of glucose and insulin, is accompanied only by slight increase in serum calcium. When calcium chloride solution and parathyroid extract are injected intravenously simultaneously, the disappearance of the injected calcium is not retarded, and the subsequent rise in serum calcium in response to the parathyroid extract is not modified; this is evidence against the theory that parathyroid extract makes the blood serum a more efficient solvent for calcium compounds.

FURTHER STUDIES ON THE OCCURRENCE OF CARBHEMO-GLOBIN IN THE BLOOD

By WILLIAM C. STADIE, F. WILLIAM SUNDERMAN, HELEN O'BRIEN, and PRISCILLA WILLIAMS

(From the John Herr Musser Department of Research Medicine, University of Pennsylvania, Philadelphia)

A continuation of the studies previously reported before the Society on solutions of pure hemoglobin has been made. The experiments, however, have been performed upon the entire contents of red blood cells and upon whole blood.

1. Red blood cell contents were equilibrated with known tensions of CO_2 and CO_2 content and the pH determined.

2. Red blood cell contents were equilibrated with CO_2 and osmotic pressure and the total CO_2 were simultaneously determined.

3. The whole blood was equilibrated at varying CO_2 tensions and complete electrolyte analyses of both serum and cells were made.

The experimental evidence obtained by these three methods of

approach are discussed in relation to the problem of the possible existence of carbhemoglobin in blood or red blood cell content.

THE DEPRESSOR ACTIVITY OF BRAIN EXTRACTS*

By C. J. WEBER, J. B. NANNINGA, and RALPH H. MAJOR

(From the Department of Experimental Medicine, the University of Kansas School of Medicine, Kansas City)

Extracts of whole brain have a marked depressor activity in both atropinized dogs and rabbits. Extracts of other tissues prepared by the same method do not have as marked a depressor activity.

The substance in brain responsible for this activity is not precipitated by phosphotungstic acid. It is not absorbed by norit but is partially absorbed by Lloyd's reagent. It is stable to acids and alkalies.

0.20 mg. of one preparation of purified brain extract given intravenously to a dog caused a fall of 36 mm. in blood pressure. The same fall was produced by 0.01 mg. of histamine. However, no imidazole reaction could be obtained with this brain extract in carefully controlled tests. Neither did this brain extract have any effect on the isolated guinea pig uterus even when 4.4 mg. of it were used, although 0.05 mg. of histamine caused a marked contraction.

Its activity on an atropinized animal excludes choline. Adenosine or adenylic acid is excluded by its stability towards acids.

We believe that we are dealing with a substance which has not as yet been identified with any known class of depressor compounds. Whether this substance has a general distribution in other tissues we cannot at present state.

CHEMOTHERAPY OF NEW DERIVATIVES OF 3-AMINO-4-HYDROXY-PHENYLARSONIC ACID*

By GEORGE W. RAIZISS, M. SEVERAC, and LEROY W. CLEMENCE

(From the Graduate School of Medicine, University of Pennsylvania, and the Dermatological Research Laboratories, Philadelphia)

Researches in the last decade brought out the importance as therapeutic agents of pentavalent organic compounds of arsenic, among them tryparsamide, chemically known as sodium salt of N-(phenyl-4-arsonic acid)glycine amide, and acetarsone (stovar-

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* Presented before the American Society for Pharmacology and Experimental Therapeutics.

sol), 3-acetylamino-4-hydroxyphenylarsonic acid. The former has been used with success in the treatment of trypanosomiasis and late neurosyphilis, and the latter in all types of syphilis and amebic dysentery. Tryparsamide is prepared by the condensation of chloroacetamide with p-aminophenylarsonic acid. However, inasmuch as 3-amino-4-hydroxphenylarsonic acid is more closely related to arsphenamine, one of the best remedies we possess today for the treatment of syphilis, compounds derived from this should possess greater potential therapeutic value.

Only one substituted glycine amide derivative of 3-amino-4-hydroxyphenylarsonic acid is known, this being the N-(2-hydroxyphenyl-5-arsonic acid)glycine ureide prepared by Jacobs and Heidelberger. We and others have attempted to prepare further derivatives of this type, but without success, probably due to the position of the amino and hydroxy groups ortho to one another. This, of course, is an unstable arrangement and makes the compound easily susceptible to oxidation, being analogous to the behavior of o-aminophenol. Therefore, we considered the advisability of protecting the hydroxyl group, making it unreactive, and utilizing the reactivity of the amino group.

Several new products were made by us and their toxicity and trypanocidal properties studied.

ELECTRICAL CONDUCTIVITY OF HUMAN TISSES FOR ALTERNATING CURRENTS OF ONE MILLION CYCLES PER SECOND*

By ALLAN HEMINGWAY and J. F. McCLENDON

(From the Department of Physiology, University of Minnesota, Medical School, Minneapolis)

It has been shown previously that on death of an animal the electrical conductivity of muscle tissue measured with high frequency alternating currents does not change. The electrical conductivity measured with low frequency alternating current, $i.e.$ 1000 cycles per second, after death, at first increases and later decreases. Cooling the tissue after death and later bringing it back to body temperature within a few hours does not appreciably change its high frequency conductivity. This makes it probable that high frequency conductivity of freshly extirpated tissues is the same as that of the living tissue.

* Presented before the American Physiological Society.

Human tissues were obtained from the operating room or from fresh cadavers. These were quickly transferred to closed conductivity cells, brought to 37°, and the conductivity measured with alternating currents of 1,000,000 cycles per second. A high frequency Wheatstone bridge, with a heterodyne method of detection, was used for measurement of the electrical conductivity. The results show a high electrical conductivity for bone and fat with a low conductivity for muscle and the internal organs, in agreement with the results of Wildermuth and Bachem.

STUDIES ON THE RELATION OF DIET TO GOITER

I. A DIETARY TECHNIQUE FOR THE STUDY OF GOITER IN THE RAT*

By HAROLD LEVINE

(*From the Laboratory of the South Carolina Food Research Commission and the Department of Nutrition of the Medical College of the State of South Carolina, Charleston*)

In confirmation of the findings of Krauss and Monroe[37] it was found that the well known Steenbock rachitic Ration 2965 produces an enlargement of the thyroid gland in rats. An intensive study of the influence of this diet on the thyroid gland was made with the view of developing a suitable dietary technique for the study of goiter in the rat.

Since the addition of vitamin D (as irradiated yeast) and of vitamin A (as carotone) did not influence the ability of the Steenbock ration to produce goiter, the following diet was adopted as the goitrogenic ration: yellow corn 76 parts, wheat gluten 20 parts, $CaCO_3$ 3 parts, NaCl 1 part, irradiated yeast 0.2 part. The irradiated yeast was added to prevent rickets. Since yellow corn is abundant in vitamin A, no additional source of this accessory factor was included. On analysis, various batches of this ration were found to contain 15 parts per billion of iodine.

On the above diet, it was found that considerable thyroid enlargement took place in 35 days (the time usually required for vitamin D assays). This time interval was, accordingly, used in all our studies on various goiter problems.

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* Presented before the American Society for Experimental Pathology.
[37] Krauss, W. E., and Monroe, C. F., *J. Biol. Chem.*, **89**, 581 (1930).

Comparisons were made with rats receiving a stock ration (containing 42 to 72 p.p.b. of iodine) and with rats receiving the goiter-producing ration containing added iodine (400 p.p.b. of iodine). The three rations were fed for a period of 35 days to normal rats weighing 60 gm., raised on our stock diet.

Data summarized from five series of experiments yielded the following average thyroid weights (fresh) per 100 gm. of body weight: (1) goitrogenic diet (193 rats), 53.2 ±0.92 mg. (range 24.1 to 126.0 mg.), (2) stock diet (thirty-nine rats), 12.9 ±0.25 mg. (range 9.0 to 18.6 mg.), and (3) goitrogenic diet containing added iodine (twenty-four rats), 12.6 ±0.19 mg. (range 10.5 to 16.0 mg.). The extent of average thyroid enlargement was therefore approximately 4 times the normal thyroid weight, with 2.3 to 7.9 times as the extremes of enlargement. The goitrous thyroids were dark red in color in contrast to the pale pink of the normal glands. Histological examination of the goitrous glands showed marked hyperplasia with a lack of colloid.

When compared with the normal glands, the dry matter and iodine content of the goitrous thyroids was found to be low. Practically the entire enlargement of the gland is due to an increase in tissue weight.

In our goiter studies, we are using the preventive technique, placing at least ten rats on the goiter-producing ration as controls and a like number (litter mates) on the supplemented goitrogenic ration or on the diet to be studied by comparison. At the end of the 35 day experimental period, all the thyroids in a given group are pooled so as to furnish sufficient glandular material for an adequate determination of iodine content.

STUDIES ON THE RELATION OF DIET TO GOITER

II. THE IODINE REQUIREMENT OF THE RAT*

By ROE E. REMINGTON

(*From the Laboratory of the South Carolina Food Research Commission and the Department of Nutrition of the Medical College of the State of South Carolina, Charleston*)

The goitrogenic ration referred to in the previous communication contained 15 parts per billion of iodine, yielding an average

daily iodine intake of 0.14 γ (1.0 γ = 0.001 mg.).   Using this diet as a basal ration, various diets were prepared, containing added iodine in the form of KI.

In the first series of experiments, five diets furnishing different amounts of iodine were fed to groups of rats containing ten animals in each group.   The rations furnished average daily iodine intakes of 0.14 γ, 0.59 γ, 1.02 γ, 1.93 γ, and 3.72 γ.

In a second series of experiments, diets yielding average daily iodine intakes of 0.14 γ, 0.18 γ, 0.29 γ, 0.38 γ, and 0.48 γ were studied.

The results of these two series of experiments indicate that there is an inverse relationship between the iodine intake and the fresh thyroid weight.   On the other hand, it was found that both the dry matter and iodine content of the thyroid increased with the iodine intake.

In order to prevent enlargement of the thyroid gland in the rat, these experiments indicate that approximately 1 to 2 γ of iodine daily are necessary.   This intake of iodine yielded glands containing 0.1 to 0.2 per cent of iodine (dry basis).   Studies attempting to throw further light on the iodine requirement of the rat are in progress.

HORMONES IN CANCER

VII. EFFECT OF THE ANTERIOR LOBE GROWTH-PROMOTING PRINCIPLE ON THE GROWTH BEHAVIOR OF NEOPLASMS*

By FRITZ BISCHOFF, L. C. MAXWELL, AND H. J. ULLMANN

(*From the Department of Cancer Research, Santa Barbara Cottage Hospital, Santa Barbara*)

The present study extends our findings previously reported concerning the accelerating effect of the pituitary growth-promoting principle upon tumor growth.   Standardized preparations were injected daily into rats inoculated with a sarcoma or a carcinoma and into mice bearing a spontaneously developed carcinoma or inoculated with a carcinoma, a suitable number of animals in each series serving as controls.

In young rats with rapidly growing tumors it was difficult to show the accelerating action on tumor growth, but in older ani-

* Presented before the American Society for Experimental Pathology.

mals, in which the body growth plateau had been reached, and in the case of slow growing tumors, the acceleration of tumor growth following the administration of the hormonal preparation was marked.

The effect of administration of the active principle was rendered more striking following intensive Roentgen ray irradiation of the pituitary region of young animals, a procedure which is shown to inhibit the activity of the gland.

The acceleration of tumor growth was not dependent upon the general growth effect on the body as a whole, for positive results were obtained under experimental conditions in which the body weight of dosed rats remained constant in contrast to controls which gained weight.

STUDIES IN KIDNEY INSUFFICIENCY PRODUCED BY PARTIAL NEPHRECTOMY; LIVER DIETS*

By ALFRED CHANUTIN

(*From the Biochemical Laboratory, Department of Medicine, University of Virginia, University*)

Diets containing 20, 40, 60, and 80 per cent whole dried liver have been fed to partially nephrectomized white rats in an effort to study the so called nephrotoxic action of these materials. In addition a diet rich in the water-soluble liver extractives and a diet rich in the liver residue left after water extraction have been fed. Our results based on functional tests, including kidney concentration tests, growth, blood studies, and blood pressure measurements, seem to indicate that whole liver and its fractions are not toxic to an already overloaded kidney tissue.

IS THERE A COMPOUND OF THE NATURE OF CALCIUM CITRATE PRESENT IN THE BLOOD?

By DAVID M. GREENBERG AND LOUIS GREENBERG

(*From the Division of Biochemistry, University of California Medical School, Berkeley*)

The hypothesis that a considerable fraction of the diffusible calcium of the blood exists as a complex negatively charged ion in a citrate-like combination with some unknown organic substance

* Presented before the American Society for Experimental Pathology.

has found a considerable number of supporters.   To a large degree this hypothesis rests upon the results of electrodialysis experiments on serum carried out by Bernhard and Beaver[38] and Klinke[39] and of adsorption experiments on serum with calcium phosphate and bone by Klinke[39] and von Beznák.[40]   Similar electrodialysis and adsorption experiments on blood serum, serum ultrafiltrates, and inorganic solutions carried out by us show that the conclusions of the above authors are erroneous.   Our results lead to the conclusion that the presence of such a hypothetical calcium compound in the blood cannot be experimentally demonstrated by any of the procedures that have been proposed.

THE IODINE CONTENT OF CERTAIN PATHOLOGICAL BLOODS IN A GOITROUS REGION (DETROIT, MICHIGAN)

By R. G. TURNER

(*From the Department of Medical Research, Detroit College of Medicine and Surgery, Detroit*)

The investigation includes a report on the iodine content of 101 pathological human bloods.   The bloods of 67 diabetics, fifteen cases of hyperthyroid, four of hypothyroid, and fifteen miscellaneous cases were determined for iodine by the micro colorimetric method of the author.

The results found show that 36 per cent of the diabetic cases averaged 11 γ per cent iodine, 34.7 per cent averaged 6.9 γ per cent, and 29.3 per cent averaged 44.6 γ per cent.   Of the hyperthyroid cases 33.3 per cent averaged 11.5 γ per cent and 66.7 per cent averaged 86.8 γ per cent.   All of the hypothyroid cases fell within the normal range, 9 to 15 γ per cent.   Of the miscellaneous cases 60 per cent averaged 11.6 γ per cent, 26.7 per cent averaged 6.1 γ per cent, and 13.3 per cent averaged 66.8 γ per cent.

High blood iodine values in diabetic cases indicate hyperthyroidism accompanying or preceding the onset of clinical diabetic symptoms.   High iodine findings in cases with no symptoms of thyroid disfunction indicate a chronic systemic infection.   Subnormal values are not considered as pathological but the result of lowered iodine content of the diet.

[38] Bernhard, A., and Beaver, J. J., *J. Biol. Chem.*, **69**, 113 (1926).

[39] Klinke, K., *Biochem. Z.*, **213**, 177 (1929).

[40] von Beznák, A., *Biochem. Z.*, **223**, 295 (1930).

In exophthalmic goiter the blood iodine ranges from 2 to 3 times the normal. In toxic adenoma accompanied with hyperthyroidism it ranges from 5 to 20 times the normal. The increase apparently depends on the degree of toxicity of the goiter.

THE MECHANISM OF BASE CONSERVATION OF RATS RECEIVING A DIET LOW IN INORGANIC CONSTITUENTS

By RICHARD O. BROOKE and ARTHUR H. SMITH

(From the Laboratory of Physiological Chemistry, Yale University, New Haven)

In previous studies[41, 42] pertaining to the growth of rats on diets deficient in inorganic constituents, a diet was prepared consisting of purified casein, hydrogenated fat, and dextrin, and containing an extremely low percentage of ash. Smith and Swanson[42] succeeded in maintaining experimental animals at a weight of about 160 gm. over a period of 90 days, despite the stringent dietary deficiency in mineral matter; some of the animals have lived for 150 days. This is all the more remarkable when it is realized that the diet is potentially an acid-producing one, extremely poor in fixed base, and that these animals were able to overcome successfully the excess of phosphoric and sulfuric acids which must be produced in the process of protein metabolism within the organism.

A group of rats grown under the conditions described by Smith and Swanson[42] was maintained for 90 days and collections of feces and urine made at 21 day intervals. A modification of the technique advocated by Mitchell[43] was employed for this purpose. Complete balance experiments were carried out on nitrogen, calcium, phosphorus, and chlorine and the excretion of fixed base and ammonia in the urine was studied. A few typical results are presented here; only those possessing an interest in connection with the conservation of base by the experimental animals are given.

Table I indicates the mechanism brought into play by the organism whereby the essential conservation of base is maintained. Urinary ammonia is excreted by the experimental animals far in

[41] Smith, A. H., and Schultz, R. V., *Am. J. Physiol.*, **94**, 107 (1930).

[42] Smith, A. H., and Swanson, P. P., *Am. J. Physiol.*, **90**, 517 (1929).

[43] Mitchell, H. H., *J. Biol. Chem.*, **58**, 873 (1923–24).

excess of that of the normal controls.   The acidity of the urine of these rats has increased to a very considerable extent.   There is therefore a much more effective removal of acid from the blood by the kidneys.   This is obviously made possible by a significant increase of urinary phosphorus.   Fortunately ample phosphorus was supplied to the animals receiving the low ash diet because of the use of casein as a source of protein.   Furthermore, the content of phosphorus in the vitamin adjuvants is relatively high. As much as 6 mg. per day, or about 30 per cent of the total intake of this element, was supplied in this way.

TABLE I

pH of urine		Percentage of absorbed phosphorus excreted in urine		Ammonia and fixed base in urine, cc. 0.1 N NaOH			
				Normal controls		Experimental rats	
Normal controls	Experimental rats	Normal controls	Experimental rats	Ammonia	Fixed base	Ammonia	Fixed base
6.7	5.9	61	89	14*	122	54	6
6.7	5.9	72	87	10	147	47	7
6.7	6.0	61	85	12	138	47	6
6.8	6.0	64	85	9	150	48	11

* Quantity for 6 day period.

THE METABOLISM OF CYSTINE AND METHIONINE

By RICHARD J. BLOCK and RICHARD W. JACKSON

(*From the Laboratory of Physiological Chemistry, Yale University, New Haven*)

Contrary to the report of Sullivan, Hess, and Sebrell,[44] we have found that the administration of β,β'-diaminodiethyldisulfide dihydrochloride has no appreciable effect on the growth of albino rats ingesting a diet deficient in cystine.   The substance tested was a beautifully crystalline and colorless preparation with a melting point of 215° (corrected) and had the following composition.   Calculated, C 21.31, H 6.26, N 12.44, Cl 31.49.   Found, C 21.77, H 6.21, N 12.05, Cl 31.30.

Other experiments show that benzyl-S-cysteine, methyl-S-cys-

[44] Sullivan, M. X., Hess, W. C., and Sebrell, W. H., *Pub. Health Rep., U.S.P.H.S.*, **46**, 1924 (1931).

teine, ethyl-S-cysteine, and benzoyl-N-cystine do not supplement the basal ration. As might be expected, cystine methyl ester hydrochloride is physiologically available in lieu of cystine. Preliminary tests seem to indicate that γ-methio-α-hydroxybutyric acid as the zinc salt causes the resumption of growth in animals subsisting on the diet poor in cystine.

A METHOD OF COLLECTING AND PRESERVING SMALL BLOOD SAMPLES FOR GLUCOSE DETERMINATIONS

By MARY E. EWING

(From the Biochemical Laboratory of the Metropolitan Life Insurance Company, New York)

A practical method has been devised for collecting small samples of blood and preserving their glucose content. The samples are collected and kept in capillary pipettes sealed by means of rubber bands. As a preservative the pipettes contain a small amount of sodium fluoride and mercuric chloride, introduced in the form of a suspension solution. Blood samples may be kept from 1 to 14 days or until evaporation begins. In a large majority of cases the glucose value will remain constant within an error of 5 per cent.

THE PROTEIN REQUIREMENTS OF THE ALBINO MOUSE

By FRANKLIN C. BING, W. L. ADAMS, and R. O. BOWMAN

(From the Department of Biochemistry, School of Medicine, Western Reserve University, Cleveland)

Young mice were fed complete diets of the synthetic type in which the protein (casein) level was 0.95, 1.96, 4.0, 7.8, 15.6, 23.2, 49.7 per cent of the total calories ingested. The food consumption and changes in body weight were measured between the ages of 3 and 7 weeks. All the animals ate approximately equal quantities of food, except those fed the lowest and highest levels, respectively, where the intakes were less, though perhaps not significantly so. The mice fed 0.95 per cent casein lost weight, those fed 1.96 per cent maintained weight, and those fed 4.0 per cent grew at a definitely subnormal rate. The mice fed 15.6, 23.2, and 49.7 per cent casein grew at nearly identical rates and attained almost the weight of animals fed upon a stock diet. Mice fed upon 7.8 per cent casein grew at a slightly subnormal rate.

The reduced glutathione concentration (Hess' modification of Okuda's method) in the muscles was about the same in all animals that grew, even though the growth might be stunted.   The mice fed 7.8 per cent casein had about half as much glutathione in the liver as mice fed higher amounts of casein, indicating that their growth, which was also the most efficient in terms of gain in weight per gm. of ingested casein, was under a condition of nutritional stress.   It is concluded that, contrary to what has been believed, the protein requirements of the mouse are certainly fulfilled by diets containing 15.6 per cent casein, and probably somewhat less would also be satisfactory.   These are about the same as the requirements of the rat, the variations due to differences in metabolic rates becoming evident when the gain in weight per gm. of ingested protein is computed.   Thus, 1 gm. of casein will produce less than 1 gm. of mouse, or about 2 gm. of rat.

THE MALE HORMONE

V. THE EFFECT OF THE MALE HORMONE AND THE ANTERIOR PITUITARY

By CASIMIR FUNK and BENJAMIN HARROW

(From the Casa Biochemica, Rueil-Malmaison, France, and the Department of Chemistry, College of the City of New York, New York)

Using rats about 1 month old, we divided them into four groups: (a) controls, (b) injected with male hormone (using the equivalent of 60 to 70 cc. of urine per day), (c) injected with the anterior pituitary (using twice daily portions of 0.3 cc. of the urine of pregnant women), (d) injected both with the male hormone and the anterior pituitary combined.   The animals were killed at the end of 4 days and their seminal vesicles examined.   The controls showed not only extremely small seminal vesicles, but there was very little glandular development, and hardly any secretion. The seminal vesicles of the animals injected with the male hormone were somewhat larger and they contained more secretion.   Somewhat larger were the seminal vesicles of the animals injected with the urine of pregnant women, and they showed a little more secretion.   In the rats that received both the male hormone and the urine of pregnant women, the pictures of the seminal vesicles were outstanding; the organs were large, there were many ramifications of the glandular structure, and there was much secretion.

STUDIES ON THE GUANIDINE CONTENT OF HUMAN BLOOD

By JEROME E. ANDES and VICTOR C. MYERS

(From the Department of Biochemistry, School of Medicine, Western Reserve University, Cleveland)

The guanidine-reacting substances of the blood have been studied in a series of thirty-six normals and a large variety of pathological conditions. A modification of the procedure described by Pfiffner and Myers[45] was employed. The guanidine substances in the blood of normal males ranged from 0.20 to 0.28 mg. per 100 cc. of blood, with an average of 0.24 mg., while for females the range was 0.18 to 0.27 mg., with an average of 0.23 mg.

Blood guanidine determinations were made on forty-nine cases showing nitrogen retention, with blood urea nitrogen figures ranging from 15 to 311 mg. per 100 cc. The guanidine values ranged from 0.28 to 2.65 mg. Although the nitrogen retention was the result of a variety of renal disorders, the guanidine values, in general, paralleled the degree of nitrogen retention, as measured by the urea and creatinine. The blood guanidine values were not proportional to the blood pressure. Some of the cases with the highest values had a normal blood pressure. However, the cases with high blood pressures had slightly higher guanidine values than the cases with a normal blood pressure with the same degree of nitrogen retention. In only one out of twelve cases of hypertension without nitrogen retention was the blood guanidine elevated above normal. The results of our work indicate that increased blood guanidine in hypertension is a matter of renal retention, and thus would be a result rather than a cause of any hypertension present.

Although the blood guanidine has been studied in a variety of pathological conditions, the findings in most of these were normal. A study of several cases of parathyroid tetany suggests the possibility of an increase in the blood guanidine in severe cases. Cirrhosis of the liver showed slightly increased values, but generally this increase was accompanied by some nitrogen retention. In a study of thirty-eight cases suffering from toxemias of pregnancy, a rise in the blood guanidine was frequently found to occur both before and after delivery. A rise in the blood guanidine of normal postpartum maternal cases was also observed.

[45] Pfiffner, J. J., and Myers, V. C., *J. Biol. Chem.*. **87**. 345 (1930)

There is no definite evidence that the substance referred to is guanidine or any of its derivatives, but it behaves as guanidine or a simple guanidine derivative other than creatine or creatinine.

THE UNMASKING OF ARGININE IN PEPTIC DIGESTION

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Peptic hydrolysis of casein, under the conditions imposed, results in the unmasking of 20 per cent of the total arginine in 15 days; 30 per cent in 30 days. Under the same conditions gelatin yields 5 per cent of its arginine in 15 days; 7 per cent in 30.

Aliquots of these digests, adjusted and exposed to trypsin, yield 80 per cent of the arginine in 15 days, in both proteins. We thus confirm Hunter's finding that 80 per cent of the arginine of these proteins is unmasked by trypsin alone. Pepsin evidently opens no protein bonds involving arginine, which are not opened by trypsin. Trypsin, on the other hand, opens many more arginine complexes than does pepsin, and the two proteins are very unlike in their content of pepsin-labile bonds. 20 per cent of the total arginine in both proteins is in a form resistant to both pepsin and trypsin, acting separately or in series.

We may therefore represent the distribution of arginine in these proteins as follows:

Protein	Total arginine, per cent		
	Pepsin-labile	Trypsin-labile	Resistant
Casein..................	30	80	20
Gelatin...............	7	80	20

THE CHEMICAL STUDIES OF TOAD POISONS

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The chemical investigation on toad poisons has been continued. the secretion of different species of toads the various prin-

ciples have been isolated and may be classified in three main groups: (1) bufotenines, (2) bufagins, and (3) bufotoxins.

The bufotenines are obtained as flavianate salts. Physiologically these compounds raise the blood pressure and stimulate smooth muscle. Chemically some of them seem to be derived from β-indoleethylamine. Wieland and his coworkers[46] think that the bufotenines obtained from Ch'an Su and the secretion of *Bufo vulgaris* are closely related to the betaine of tryptophane. It seems to us rather doubtful that compounds of such chemical structure should possess blood-raising properties. We have found that the betaine of tryptophane has no action on the blood pressure. This does not support the expressed view of Wieland and his coworkers.

The bufagins have a physiological action on the heart similar to that of digitalis, but the action is less persistent than that of digitalis. They are stable in neutral solution, but decompose on being heated in alkaline or acid solution. They are unsaturated neutral compounds and contain a lactone group and several hydroxyl groups, one of which is linked to an acid; *e.g.*, formic acid or acetic acid.

The bufotoxins have a physiological action similar to that of the bufagins. They are stable in neutral solution but decompose on being heated in alkaline or acid solution. The molecule of the bufotoxins is made up of the corresponding bufagin linked to suberylarginine.

Pharmacological findings make it appear very probable that the toad poison principles are chemically very closely related to the cardiac aglucones of the plant kingdom. The empirical formula now assigned for cinobufagin, the principle from Ch'an Su is $C_{25}H_{32}O_6$; and for bufagin, the principle of the secretion from *Bufo marinus*, $C_{24}H_{32}O_5$. From the chemical behavior of these substances one can conclude that cinobufagin contains a lactone, an acetoxyl, a secondary hydroxy, and a tertiary hydroxy group, and is of an unsaturated nature; and that bufagin contains a lactone, a formoyl, and a tertiary hydroxy group, and is also of an unsaturated nature. By splitting off the acid radical which is attached to a hydroxy group (acetic acid from cinobufagin and

[46] Wieland, H., Hesse, G., and Mittasch, H., *Ber. chem. Ges.*, **64**, 2099 (1931).

formic acid from bufagin), one obtains compounds which are C_{23} derivatives, as are the aglucones of the plant glucosides. While the cardiac poisons of the plant kingdom are combined with carbohydrates, these principles of toad poisons are coupled with acetic acid or formic acid. Researches are in progress to ascertain whether a direct proof of the chemical relationship between these two groups of natural compounds can be found.

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[52] Waugh, W. A., and King, C. G., *J. Biol. Chem.*, 97, 325 (1932).